IN VITRO PROGESTERONE METABOLISM BY AVIAN TESTICULAR TISSUE

by

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A thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
Department of Biological Chemistry

University of Utah
August, 1961

This Thesis for the Ph. D. Degree

by

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has been approved

Chairman, Supervisory Committee

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Supervisory Committee

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to Dr. Kristen Eik-Nes for his advice, encouragement, and constant support during the course of these investigations. Special thanks are due Dr. J. R. King for providing laboratory facilities, equipment, and his time and interest, which greatly aided this work.

The assistance of Mssrs. R. B. Billiar, R. D. Dagon, W. J. Bowman, and R. Higgins in trapping and sacrificing animals is gratefully acknowledged, as is the help of my wife in the final experiment and in proof reading the manuscript.

This work was performed under the tenure of a National Heart Institute predoctoral fellowship HF-9392, and this financial support was very much appreciated.

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I. INTRODUCTION

Members of the class Aves have, by no means, been ignored in the field of biochemistry. The pigeon breast muscle and liver have been extensively used in metabolic studies. Physiologically perhaps as much is known about avian endocrinology as mammalian. However, as the field of endocrinology has gradually reached a biochemical level over the past thirty years, knowledge of the biochemistry of mammalian endocrine glands has greatly surpassed that of avian glands. This situation is exemplified by the comparative state of knowledge of the androgenic hormones and testicular function. Thus, the chick comb bio-assay for androgens was instrumental in isolation of the first chemically pure androgen (1), and the sparrow beak assay for androgens has been recognized since 1933 (2). These and other physiological criteria led Dorfman (3) to define androgens as "compounds which stimulate secondary sex structures of mammals and birds." But the biochemical knowledge of androgenic compounds in birds is virtually non-existant.

An interesting phenomenon of avian testicular function that has received a great deal of scrutiny from experimental zoologists and physiologists is the pronounced annual cycle in many species of Temperate Zone birds. Here again, the physiological aspects of testicular cycle have been thoroughly investigated, but the biochemical

behavior of testis tissue during this cycle is not known.

These were the questions that were asked at the beginning of this study: What androgenic hormone(s) does the avian testis produce, or what androgenic hormone(s) is it capable of producing? How does the biosynthetic pathway from progesterone to androgens compare with that found in mammalian testicular tissue? How does the capacity of the testis to produce androgens vary in the pronounced annual cycle of testicular size and spermatogenic activity?

The approach chosen was to perform in vitro incubation studies with sparrow testicular tissue and C^{14} labeled substrates. Since progesterone is a known precursor of androgens in mammals and has been tentatively isolated from testicular tissue of pigeons (4), it was felt that this would be a logical precursor with which to start. The obvious limitation of in vitro studies such as this is that no light is shed on the nature of the hormone secreted by the gland, but only on what the gland is capable of producing from a known precursor. However, from the viewpoint of comparative biochemistry this is perhaps the more interesting aspect.

II. HISTORICAL REVIEW

A. Androgens. The effects of removing the testicles from an animal have been recognized for almost as long as there have been written records. Thus, castration of slaves in ancient Greece was practiced to produce more docile and trustworthy servants for household duties, and males with soprano voices were produced by this method for service in the papal choir of Rome. In about 300 B. C. Aristotle (5) recorded the effects of castration in the chicken. He observed that loss of testicular function in the adult bird caused comb regression, cessation of crowing, and loss of sexual desire, while similar treatment of the young bird prevented any of these characters from developing. The analogy between these effects and those observed in man was drawn. Hunter (6) was perhaps among the first to record the specific effects of extirpation of the testicles on the structure of the secondary sex organs in mammals. He observed that the penis, seminal vesicles, and prostate all depended on the presence of the testes for their normal structure, but that unilateral castration did not cause the atrophy of the seminal vesicles.

The true endocrine function of the male gonads was not unequivocally demonstrated until more than 50 years after Hunter's observations. In 1849 Berthold reported the results of testicular transplantation

experiments in chickens (7). He adequately showed that the secondary sex characteristics of the cock were maintained even if the testes were in an abnormal location and thus deprived of their normal nervous connections. This was ample evidence that the male gonad was secreting a "hormone", as defined half a century later (8). This work gave impetus to attempts of preparing testicular extracts containing the masculinizing principle(s). Although such preparations had been used as aphrodisiacs as early as the 8th century (9), it was not until the latter part of the 19th century that the effects of injection of testicular extracts in male animals was recorded in scientific literature. Brown-Sequard (10), Pregl (11), and Zoth (12) all reported the rejuvenation effects following self-injection with aqueous or glycerol extracts of testicular tissue. These observations were extremely subjective, as recognized by the authors, and were, at best, only suggestive in nature.

More empirical evidence for preparations containing substances affecting male secondary sex characters accumulated in the first two decades of the 20th century. Loewy (13) in 1903 showed effects of aqueous testicular extracts on capon comb growth, while Ancel and Bouin (14) demonstrated the capabilities of similar preparations to increase seminal vesicle size in castrated guinea pigs. In 1908

Walker (15) confirmed the earlier observations of Loewy, but used cockerel rather than mammalian testes as tissue source. Pézard observed that extracts of gonadal tissue from a cryptorchid hog would also produce comb growth and sexual behavior in chickens (16). All of the extracts used by investigators to this time were either aqueous or glycerol and had a very low activity. Injections were continued over periods of 5-9 months in order to demonstrate positive effects.

Following the lead of Doisy (17) and Gustavson (18) isolating estrogens, Korenchevsky (19) and McGee et al. (20) independently prepared lipoid extracts of bovine testis tissue. Korenchevsky's results were rather dubious, while those of McGee, possibly because of a more sensitive bio-assay, were quite convincing, since he was able to produce considerable comb growth in the capon by injections of purified material. Whereas previous workers had required several months to show the effects of non-lipoid extracts on secondary sex characters, McGee obtained a positive reaction within five days. Gallagher and Koch purified McGee's material to the point that the minimum effective daily dose was a mere 10 μgm (21). Active material was subsequently prepared from swine and stallion testes (22).

It would seem that these testicular androgenic hormones were of quite high purity. However, the first crystalline androgen, androsterone,

was obtained in 1931 from urine by Butenandt (1), after Loewe and coworkers reported androgenic activity in extracts of this fluid (23). The structure was proposed a year later (24) and subsequently proved by partial synthesis by Ruzicka in 1934 (25). Ensuing work showed the androgenic material in testis extracts to be more active than the crystalline urinary androgens in stimulating growth of rat seminal vesicles. Dingemanse and coworkers (26) isolated a much more active crystalline androgenic hormone, testosterone, from testicular tissue after Gallagher and Koch (27) had shown that alkali inactivated the testicular but not the urinary hormone. Proof of the structure of testosterone was provided independently by two groups in Germany (28, 29) by partial synthesis. Later, Ruzicka also isolated testosterone from stallion and boar testicular tissue (30, 31). A second Δ^4-3-keto-androgen, 4-androstene-3, 17-dione (androstenedione), had been prepared synthetically from cholesterol (32) but was not isolated from natural sources until 1941, when von Euw and Reichstein reported its presence in adrenal cortical extracts (33). Lieberman then reported its isolation from urine of a patient with adrenal hyperplasia (34).

Since testosterone was the most active androgenic steroid and had been isolated from testicular tissue of several species, it was generally assumed that this was the hormone secreted by the testes. Except for some amazing results obtained by Busquet (35),

there was no direct evidence for the occurence of androgenic material in circulating body fluids. Busqet reported comb growth, crowing and treading in capons fed 5 ml of bull, stallion, or ram blood daily for 7-10 days. However, it was not until West isolated testosterone and androstenedione from spermatic vein blood of dogs (36) that any definitive proof existed for the secretion of these androgens by the male gonads. More recently Oertel and Eik-Nes identified testosterone in human peripheral plasma of male subjects treated with human chorionic gonadotropin (HCG) (37).

The evidence for the cellular source of androgens within the testis began with the suggestion of Loisel that the lipid granules observed in the interstitial cells were related to the internal secretion produced by this gland (38). Ancel and Bouin (39, 40) showed that ligation of the vas deferens in mammals caused atrophy of the seminiferous tubules without affecting the interstitial cells or the secondary sex characters of the animals, and Massaglia repeated these observations in chickens (41). Pézard obtained androgenically active extracts from cryptorchid hog testes that consisted primarily of interstitial cells (16). X-ray treatment of rat testes was shown to destroy the germinal epithelium and reduce the testes to 25% of their original weight (42). The interstitial cells appeared undamaged, however, and the secondary sex glands were not affected. Bucheim found that administration of fuel

oil to four species of laboratory mammals caused destruction of the testicular Leydig cells with concurrent atrophy of the seminal vesicles (43). The seminiferous tubules were not visibly damaged by this treatment. It has also been demonstrated that in a patient with an interstitial cell testicular tumor, the urinary and plasma 17-keto-steroids are markedly elevated (44). These results strongly indicated that the testicular Leydig, or interstitial, cells were producing the internal secretion of the testes.

The regulation of testicular function by the anterior pituitary was first clearly demonstrated by the transplantation of anterior pituitary material into immature rats by Smith and Engle (45). Later, Smith showed that both the interstitial and tubular elements of the testes were stimulated by the pituitary implants (46).

Evidence accumulated by Zondek and Ascheim (47-51) indicated that gonadal function was apparently regulated by two distinct activities which could be separated from female urine. Prolan A (FSH) was obtained from urine of castrate or post-menopausal women and caused follicular growth of the ovaries. Prolan B (HCG) was isolated from urine of pregnant women and caused ovulation and luteinization. Smith and coworkers (52, 53) extended Zondek's observation to the male rat where it was shown that HCG stimulated primarily the interstitial cells, and the urinary follicle stimulating hormone (FSH) caused testicular

hypertrophy without stimulating the secondary sexual characteristics and therefore, presumably not the Leydig cells.

Two hormones similar to those found in urine were separated from anterior pituitary extracts (54). Regulation of both ovarian and testicular function by these preparations was similar to that of the urinary gonadotropins (55). Evidence from several groups of workers indicated that the actions of pituitary gonadotropins and of the pregnancy urine gonadotropin were different in both mammals (55-60) and birds (59, 60), and that the pregnancy urine hormone (HCG) was similar to that obtained from placenta extracts (61, 62). Thus, HCG was shown to stimulate primarily the interstitial cells in the mammalian testis (53), as did pituitary luteinizing hormone (LH) preparations (55). However, the augmentation effect on ovarian follicular growth observed when pituitary follicle stimulating hormone (FSH) and LH were administered together was not observed with combinations of FSH and HCG.

More recent studies have shown that the perfused dog testis responds to HCG stimulation by increased production of testosterone within half an hour after the gonadotropin administration (63). The in vitro action of HCG to increase the incorporation of acetate into testosterone when incubated with testis tissue slices was reported by Brady (64), and Marsh et al. found both HCG and LH increased the in vitro synthesis

of progesterone by bovine corpus luteum slices (65).

That the avian testis is also controlled by pituitary hormones has been shown for pigeons (60, 66), ducks (59), chickens (67), and sparrows (68). All of these investigations showed the avian testis to be sensitive to injections of extracts of mammalian pituitary tissue. In addition the chicken's gonads were sensitive to homeoplastic implants of pituitary glands (69). In the majority of these cases effects were observed on both germinal tissue and secondary sex characters indicating stimulation of both the gametogenic and hormonogenic testicular functions. With the availability of more highly purified gonadotropin preparations it was demonstrated that mammalian pituitary LH stimulated primarily the chicken testis interstitial cells, while the primary effect of FSH from the same source was on the seminiferous tubules (70-72). Breneman (7 also found the chicken testes to be sensitive to HCG, while Asmundson reported these glands were stimulated by a gonadotropin preparation from pregnant mare serum (PMS) (73). Both of these latter hormones appeared to have primarily an LH effect in the chicken. Pfeiffer and Kirschbaum showed PMS injections in male sparrows caused appearance of Leydig cells with a concomitant stimulation of secondary sex characters (74). Some years later it was demonstrated that fractionation of cock pituitary extracts by a method used to prepare LH and FSH fractions

from sheep pituitaries (54) yielded preparations which were more active in the hen than were similar mammalian preparations (75, 76). Thus, the LH fraction induced, while the FSH inhibited, ovulation. This supported Witschi's earlier findings of gonadotropin activity in crude acetone powder of turkey pituitaries (77).

B. Avian testicular cycles. The annual cycles of reproductive ability and, therefore, gonadal function in birds were apparently recognized by Aristotle (78). Although such cycles occur in both male and female birds, the testicular cycle has been studied more thoroughly.

Hunter (6) actually published figures in the 18th century illustrating the great changes in size of the testicles of the English sparrow (Passer domesticus). In general these testicular cycles may be summarized as follows. The testis size is at a minimum in winter; begins to recrudesce in late winter and early spring; reaches its maximum weight in late spring and early summer, during the breeding season; begins to regress in mid-summer; returns to its minimum size and weight in early fall; and remains at this stage until the start of another cycle. The increase in testis size from winter to spring or summer may be around 200 fold in P. domesticus (79) to 1500 fold in the starling (Sturnus vulgaris) (80). A typical annual cycle is illustrated in Figure 1.

The idea that such a cycle must be externally controlled by environmental factors is probably as old as the original observations themselves. The first investigations into this control were primarily concerned with the mechanism of avian migration, since the coincidence of vernal migration and testicular recrudescence followed by autumnal

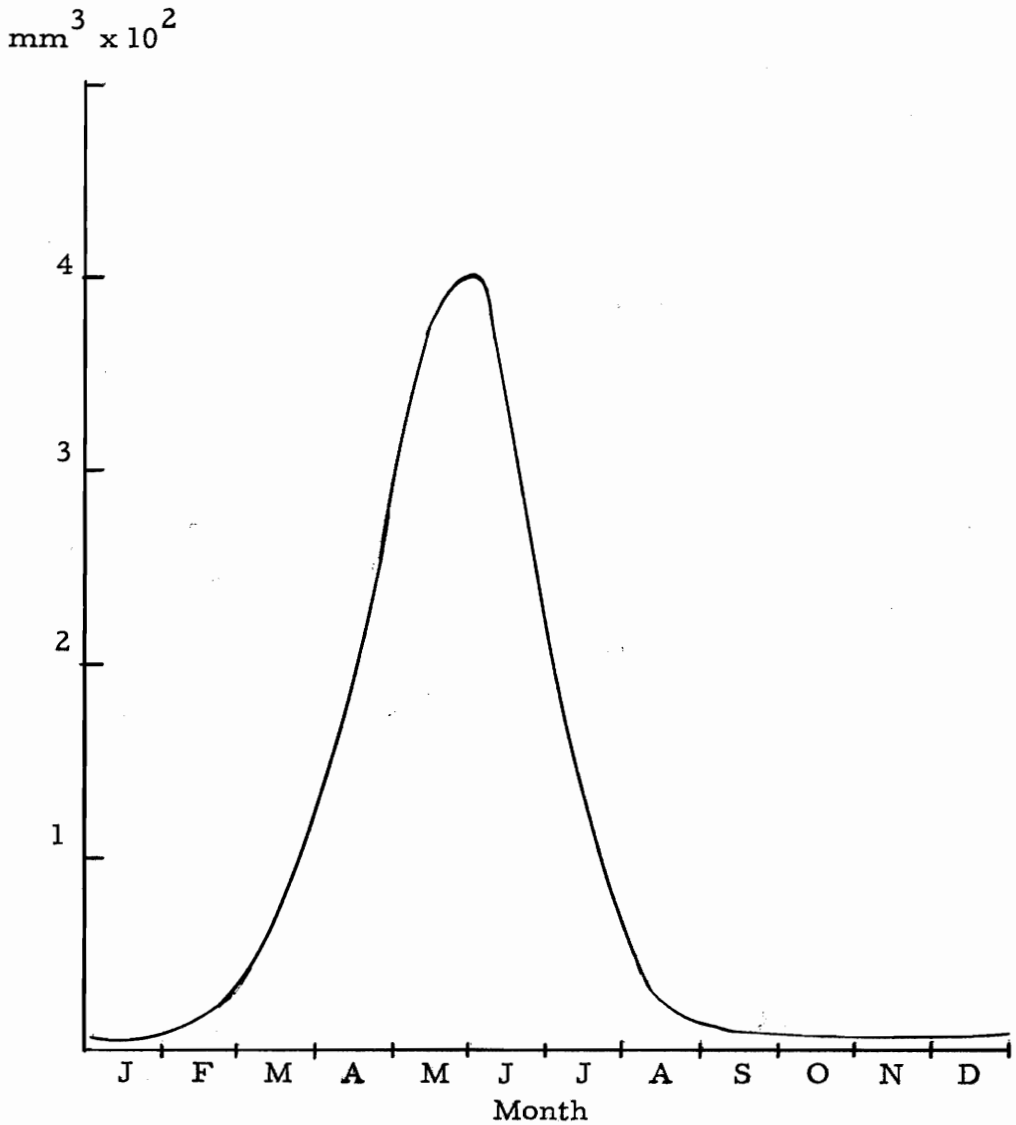


Figure 1. Typical annual testicular cycle of Passer domesticus.
Modified from Threadgold (79).

migration and testicular regression appeared too striking to be merely fortuitous. Schäfer (81) and Etzold (82) were, perhaps, among the first to clearly state the possible relationship of photo-period to the annual gonadal cycle. Experimental control of this annual testicular activity has actually been practiced for many centuries in Japan and the Netherlands, at a practical level, to produce males of the species which would sing in an "off" season (82). However, the first experimental manipulation of the cyclic phenomena on a scientific level was performed by Rowan in 1925 (83). In a series of further studies this investigator (84-87) subjected juncos (Junco hyemalis) and crows (Corvus brachyrhynchos) to a short artificial daily increase in the photoperiod starting in early October. This was designed to simulate the gradual spring increase in photoperiod naturally encountered by these species. In two months time Rowan was able to show increased gonadal size and brought some of these birds into full breeding condition by the first of January. These results were confirmed and extended by Bissonnette working with S. vulgaris (80, 89-91), Cole with mourning doves (Zenaidura macroura carolinensis) (92), and Kirschbaum (93), Kirschbaum and Ringoen (94), and Ivanova (95) with P. domesticus. Since this early work the list of species which show this response to increased photoperiod has been extended to

include twenty seven (82).

The large number of investigations into the detailed mechanism of this photoperiodic response of birds are beyond the scope of this review. A few pertinent points might be mentioned. Rowan's initial investigations (83) were conducted at winter temperatures in Edmonton, Canada. Since he was able to show a response to increased light rations, it was evident that low temperatures at least had no inhibitory effect on testicular recrudescence. Later investigations (93, 96) have shown that increased ambient temperature alone will not stimulate gonadal growth if unaccompanied by prolonged photoperiod, but may modify the response to light (97, 98). That the threshold length of the daily photoperiod required for gonadal stimulation is somewhat above 10 hours has been found for several species of birds (99, 100). The effects of wave length of light used have been studied, and the orange to red end of the spectrum has been demonstrated to be the most stimulatory (101-105).

That the light was producing gonadal growth by stimulating pituitary gonadotropin secretion was, perhaps, a reasonable assumption. Definitive experimental evidence for the intermediary role of the hypophysis was published by Benoit (106). This investigator showed that implantation of pituitaries from light stimulated ducks into

immature female mice showed more gonadotropic activity than pituitaries from non-stimulated ducks. In addition, it was subsequently demonstrated that hypophysectomized ducks no longer responded to stimulatory photoperiods (107). The route by which light stimulates pituitary secretion is not too clear. But it appears that the eyes and the ocular region in general are probably the two main receptors of light (95, 108-110), while the possibility of "deep receptors" in the hypothalamic area of the brain being activated by direct penetration of light exists, at least in the duck (107). Marshall has recently presented a review of this subject, and Benoit has discussed Marshall's presentation (111).

A period during the annual cycle that has been investigated rather extensively is the so-called "refractory period." At the end of the breeding season, when the testes regress, there is a 2-3 month period in which an artificial increase in the photoperiod will produce no testicular growth. This phenomenon was observed in the case of P. domesticus by Riley in 1936 (112). It was also noted that while the adult male sparrows showed no response to a 17 hour daily photoperiod beginning on September 30, juvenile males responded to this treatment. The conclusion drawn was that if a refractory period existed in juvenile birds, its duration was not as long as in adults. It appears that, at least in some species of Temperate Zone birds, there is a requirement

for a period of decreased photoperiod after completion of the breeding season before the pituitary will respond to photo-stimulation (113-116). That it is the pituitary rather than the gonads which fails to respond during this period has been shown by two different approaches. Benoit et al. (117) observed that apparently the adenohypophysis loses its ability to secrete gonadotropin, since the gonadotropic content of this gland was increased during the period of refractoriness. These investigators have also shown that the adenohypophysis of the duck is not caused to hypertrophy by bilateral castration during this period, nor is there compensatory testicular hypertrophy after unilateral castration (118). More direct evidence that the gonads themselves are not responsible for the failure of increased photoperiods to cause testicular enlargement has been shown by gonadotropin injections. Thus, Miller obtained testicular stimulation in golden-crowned sparrows (Zonotrichia atricapilla) by injections of 50 IU of PMS daily for 4-14 days (119), and Benoit found ducks responded similarly to injections of extracts of the adenohypophysis (120).

More comprehensive reviews of photoperiodism in birds with regard to the testicular function have been published by Farner (82) and Wolfson (121).

The histological changes which take place during the testicular cycle have been studied in many laboratories. Watson in 1919 (122) and

and Stieve, as quoted by Benoit (123), found that the germinal tissue activity in testes of the greenfinch (Ligurinus chloris) and European jackdaw (Corvus monedula) followed testis size, whereas the interstitial cell content curve of the tissue appeared to be inverse to that of the testis size. However, Watson's terminology, "relative number of interstitial cells", is difficult to interpret. If he was measuring the number of such cells in one microscopic field at a given magnification, there still might have been an absolute increase per testis due to the large increase in testicular size. Both Watson (122) and Bissonnette (124) suggest transformation of interstitial cells to fibroblasts when spermatogenic activity increases and the seminiferous tubules enlarge. The latter investigator also reported the interstitial cells to be more abundant in the regressed than in the stimulated gonads of S. vulgaris but noted that this condition could have been more apparent than actual (125). Wolfson came to a similar conclusion with data from junco testes (126). Bissonnette commented on the observation that the yellow bill of the starling, which is dependent on the secretion of androgenic hormone for its color (127), became yellow in spring prior to development of spermatogenesis. Blanchard (128) also noted that the first changes in testicular histology in the spring occurred in the interstitial tissue as evidenced by a marked increase in Leydig cells.

The time of this occurrence coincided with the time flocking behavior ceased and territorial defense started. She also reported that Rowan found a similar situation in J. hyemalis. Contrary to reports on other species, Blanchard found the total volume of glandular tissue (Leydig cells) in the intertubular material increased to a peak coinciding with the peak of spermatogenic activity or testicular size. Similar results from several investigations by different workers have been summarized by Threadgold (79). Although the actual curves drawn by this author should probably be questioned to some degree, it is apparent that spermatogenic activity and number of interstitial cells both increase during the spring and reach a maximum at about the same time, and that this holds true for four different geographical locations. Marshall examined two British birds, the robin (Erithacus rubecula) and the rook (Corvus frugiligus), that display autumnal sexual behavior following the normal spring breeding season (129). It was found that while the testes were still at a minimal size and the seminiferous tubules were unstimulated, the Leydig cells had regenerated after their post-nuptial exhaustion.

In contrast to these results, which seem to strongly indicate the presence of Leydig cells in the interstitium of avian testes, and the possibility of androgen secretion by these cells, Pfeiffer and Kirschbaum (74) stated that at no time during the normal cycle could interstitial

cells be definitely identified in English sparrow testes. However, injections of mammalian gonadotropins caused appearance of interstitial cells concomitant with a stimulation of secondary sex characters.

Thus, the annual testicular cycle in many species of birds is well documented. It is apparent that similar cycles can be produced by artificial control of the daily photoperiod, provided the birds are not in a refractory period. At this time gonadal stimulation can be accomplished by gonadotropin injections. It has been demonstrated that not only the spermatogenic activity but also the hormone producing ability of the testis undergo seasonal variation as evidenced by the changes in secondary sexual characters. However, the limits of the variation in androgen production cannot be delimited by this criterion. It appears likely, as in mammals, that the source of the testicular hormone(s) is the interstitial or Leydig cells.

C. Biosynthesis of steroid hormones from progesterone. The problem of the biogenesis of steroid hormones by endocrine glands has been present in the field of biochemistry since the chemical nature of these hormones was defined. The structural relationship between these compounds and cholesterol was quite evident, and it was subsequently shown that cholesterol could, indeed, be metabolized to steroid hormones (130, 131). However, it has yet to be conclusively shown whether cholesterol is an obligatory intermediate in the biosynthetic pathway, or if it merely represents an intermediate in one possible route to the steroids (132).

In 1943 Ruzicka and Prelog isolated 5-pregnen-3β-ol-20-one (pregnenolone) from testicular tissue (133) and were confirmed by Haines and coworkers (134). It appeared that this Δ^5-21-carbon compound could be a link between the Δ^5-27- and Δ^4-21-carbon compounds. Several years later Samuels et al. demonstrated the presence in mammalian tissues of an enzyme activity that converted the Δ^5-3β-ol structure to the Δ^4-3-ketone (135). This activity was determined by in vitro incubation techniques and was not found in tissues that did not synthesize steroid hormones. With pregnenolone as substrate the product of the reaction was progesterone. Biosynthesis from progesterone to the secretory product(s) of each gland has been studied in some detail for adrenal, placenta, ovaries, and testes.

The capacity of the adrenal gland to synthesize corticosteroids was studied first, and probably in greater detail than any other gland. In a series of papers from the Worcester Foundation for Experimental Biology in Massachusetts, the ability of the adrenal to introduce the 11β-hydroxyl group into the steroid molecule was demonstrated by adrenal perfusion studies (136, 137). Supporting evidence was obtained from in vitro incubations with adrenal slices and homogenates (138-141). By fractionation of adrenal homogenates Sweat and Hayano independently reported virtually all of the 11β-hydroxylase to be located in the mitochondral fraction (142, 141).

Since Pfiffner and North had isolated 17α-hydroxyprogesterone from adrenal glands of cattle (143, 144), this compound showed promise of being involved in the synthesis of 17-hydroxylated corticosteroids. By perfusing various possible steroid intermediates through isolated adrenal glands Hecter et al. proposed the scheme shown in Figure 2 for the pathway of biosynthesis of adrenal corticosteroids (145, 146). This suggested general pathway of corticosteroid biosynthesis has withstood intensive investigation of the biosynthetic reactions in the adrenal, and the order of hydroxylation reactions in Figure 2 appears to be the preferred sequence in both perfusion and incubation studies (147-155).

Since Bloch and coworkers had found androstenedione and its

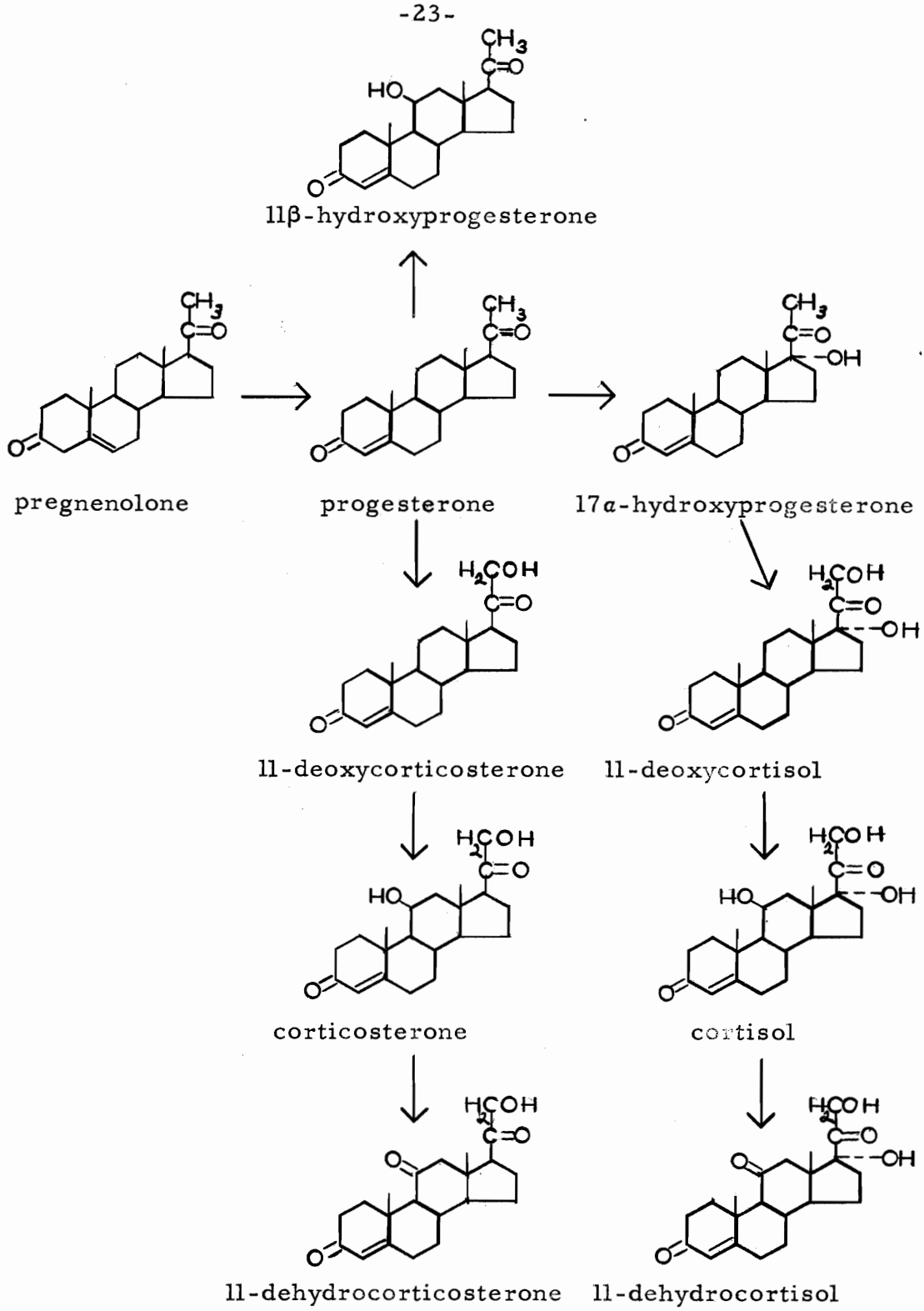


Figure 2. The pathway of adrenal steroid biosynthesis.

11β-hydroxyl and 11-keto derivatives in perfusate of bovine adrenal glands after including adrenocorticotropin (ACTH) in the perfusing medium (156), the previous observations on 11β-hydroxylation of androstenedione perfused through the adrenal gland (145, 157) and the conversion of dehydroepiandrosterone (DHEA) to androstenedione (158) appeared to have physiological significance. The ability to introduce the 11β-hydroxyl group into C-19 precursors was also found in adrenal homogenate incubations (159, 160), as was the ability to convert DHEA to androstenedione (160). Subsequently, Touchstone and coworkers (161, 162) showed that adrenal slices of a patient with severe hypertensive or metastatic malignancy produce 11β-hydroxyandrostenedione when incubated without added substrate. Bloch demonstrated the capacity of adrenal gland slices from an adrenogenital syndrome patient to synthesize DHEA, androstenedione, and 11β-hydroxyandrostenedione from radioactive acetate (163-165). Thus, the ability of the adrenal gland to synthesize C-19 steroids and the possibility of a Δ^5-3β-ol C-19 steroid being a direct precursor of the Δ^4-3-keto C-19 compounds appears likely. However, Rao and Heard have shown that hog adrenals can also synthesize androstenedione from progesterone (166).

The possible occurrence of an alternate pathway of adrenocorticoid biosynthesis, involving 17-hydroxylation of pregnenolone followed by conversion to 17-hydroxyprogesterone, was suggested in endocrine

tissue by the in vitro incubations of hamster adrenals with 17α-hydroxypregnenolone, which was converted to cortisol, corticosterone and cortisone (167). This possible intermediate, 17α-hydroxypregnenolone, has been isolated from pig adrenals (168) and from the adrenal blood of dogs after administration of ACTH (169). Information on the relative importance of this "new" pathway was obtained by Weliky and Engle by using both tritiated 17α-hydroxypregnenolone and C^{14}-labeled progesterone as substrates in the same adrenal incubation flasks (170). The ratios of tritium to C^{14} in the isolated corticoid products was little different than the ratio of the starting materials. This indicated that both pathways were operating to approximately the same extent.

In many of the in vitro incubation studies it has been found necessary to include fumarate or some other citric acid cycle intermediate in the medium to obtain maximal rates of transformation (159, 150). Sweat (171) and Grant and Brownie (172) have shown that in the case of 11β-hydroxylation this addition is necessary to facilitate reduction of triphosphopyridinenucleotide (TPN) to provide reduced triphosphopyridinenucleotide (TPNH) for the reaction. Sweat proposed the mechanism is via a transhydrogenase from the reduced diphosphopyridinenucleotide (DPNH) formed by the oxidation of malate to oxaloacetate, while Grant and Brownie suggested the TPNH is formed

via the conversion of malate to pyruvate by malic dehydrogenase.

Little work on corticosteroid biosynthesis has been done with non-mammalian vertebrates. However, deRoos has reported the isolation of corticosterone as the major secretory product after in vitro incubations of adrenals from three species of birds (173, 174). He also isolated considerable amounts of aldosterone and a minor product tentatively identified as cortisol. Under similar incubation conditions Nandi and Bern found five species of fish produced cortisol, cortisone, or both (175), while Phillips and Mulrow reported that interrenal tissue of Fundulus heteroclitus synthesized cortisol and probably cortisone from tritiated progesterone (176). In amphibians, Carstensen et al. showed that bullfrog adrenals secreted aldosterone and corticosterone in a ration of 3.6:1 when incubated in vitro (177). So while it appears that fish, bird, and amphibian adrenals synthesize and secrete corticoids identical to those synthesized by mammalian glands, there is no available information on the pathway of corticoid biosynthesis in these "lower" vertebrates.

The investigation of estrogen biosynthesis by placenta has received very little attention as compared with the biosynthesis of steroids by adrenal tissue. The most obvious reason is that from the medical standpoint the question of estrogen elaboration is not as important to the physical well-being of the individual as the question of adrenal corticoid production.

However, in 1955 Levitz and coworkers demonstrated that perfused human placenta was capable of synthesizing both estradiol and estrone from C^{14}-labeled acetate (178), and Meyer demonstrated that slices of this tissue were capable of converting both 19-hydroxyandrostenedione and androstenedione to estrone (179). The 19-hydroxylated precursor was converted to estrone at 3-8 times the rate of conversion of androstenedione to this estrogen, while no detectable conversion of 1, 4-androstadiene-3, 17-dione or of 19-norandrostenedione to estrone could be detected. The report by Longchampt et al. (180) of formation of 19-hydroxyandrostenedione and estrone from androstenedione by placental cell free homogenates is strong evidence for the role of this 19-hydroxy compound as an intermediate in the conversion of androstenedione to estrone. The subsequent isolation of progesterone and androstenedione from placental extracts (181) gave support to the possible role of these two steroids as estrogen precursors. Ryan, working with a placental microsome system, obtained a 40-60% conversion of androstenedione to estrone (182) and showed testosterone to be metabolized primarily to estradiol (183). Baggett and coworkers confirmed the conversion of C^{14}-testosterone to estrone and estradiol by placental homogenates (184).

The formation of estriol by placental microsomes and supernatant was studied by Ryan (185,186). Evidence was presented showing that 16α-hydroxyandrostenedione, 16α-hydroxytestosterone, and 5-androstene-3β,

16α,17β-triol were all converted to estriol. Since the rate of conversion of the 16α-hydroxyandrostenedione was faster than either of the other two substrates, it was proposed that 16α-hydroxyestrone was an intermediate in estriol biosynthesis from this substrate and subsequently shown that this intermediate was reduced at the 17-ketone to estriol by the preparation used. Further work demonstrated that a similar placental preparation could convert 16-ketoestrone to 16-ketoestradiol and estriol, while 16- ketoestradiol was not converted to estriol. This was substantiating evidence for the role of 16α-hydroxyestrone in estriol biosynthesis.

Just this year Little and Shaw published results of the conversion of progesterone to 17α-hydroxyprogesterone in placental tissue (187).

By pooling all results and by analogy to studies with other endocrine tissues, the most likely pathway of estrogen biosynthesis from progesterone in placental tissue is shown in Figure 3.

Ovarian hormone biosynthesis has received extensive investigation since Werthessen et al. perfused sow ovaries with radioactive acetate and isolated minute amounts of radioactive estrone, estradiol and cholesterol (188). Solomon et al. found that bovine ovarian homogenates were capable of converting progesterone to 17α-hydroxyprogesterone and androstenedione (189), after Baggett and coworkers had shown that "grossly normal" human ovarian slices formed 17β-estradiol from testosterone in vitro (190, 191). Using slices of human ovarian tissue,

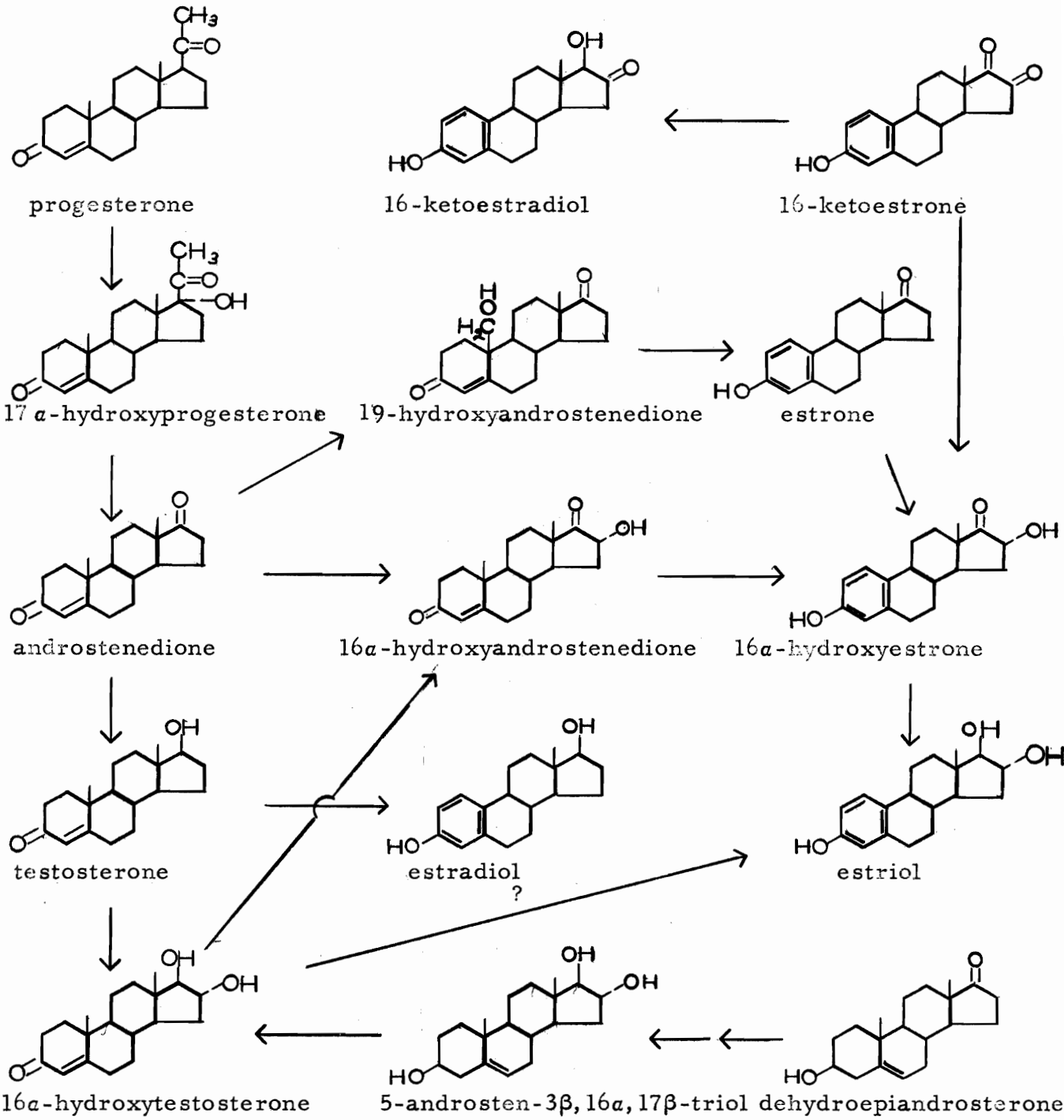


Figure 3. Estrogen biosynthesis and metabolism by placental tissue.

which showed marked cortical stroma hyperplasia, Wotiz confirmed and extended Baggett's results to include formation of estrone and estradiol from testosterone (192). In a brief communication O'Donnell and McCaig (193) reported further evidence for the general metabolic pathway of estrogen biosynthesis. In incubation studies with Stein-Leventhal syndrome ovarian slices and C^{14}-acetate, isolation of C^{14}-estrone, estradiol, androstenedione, testosterone, and 17α-hydroxyprogesterone was accomplished. In a more comprehensive report, Sweat et al. (194) found the synthesis of progesterone from acetate by both human and bovine ovarian minces. Incubations with progesterone showed the formation of 17α-hydroxyprogesterone and androstenedione, while with androstenedione substrate estrone and estradiol were formed. The general pathway of estrogen biosynthesis in ovarian tissue is shown in Figure 4.

Additional evidence and confirmation of the existence of steps of this pathway have been published by several groups in both normal and pathological ovarian preparations (195-198). This year Ryan and Smith reported the results of incubations with minces of follicular cyst linings from a woman pretreated for 9 days with FSH. They were able to show the conversion of radioactive acetate to C^{14}- estrone, estradiol and cholesterol (199), while C^{14}-progesterone was also converted to estrone and estradiol (200).

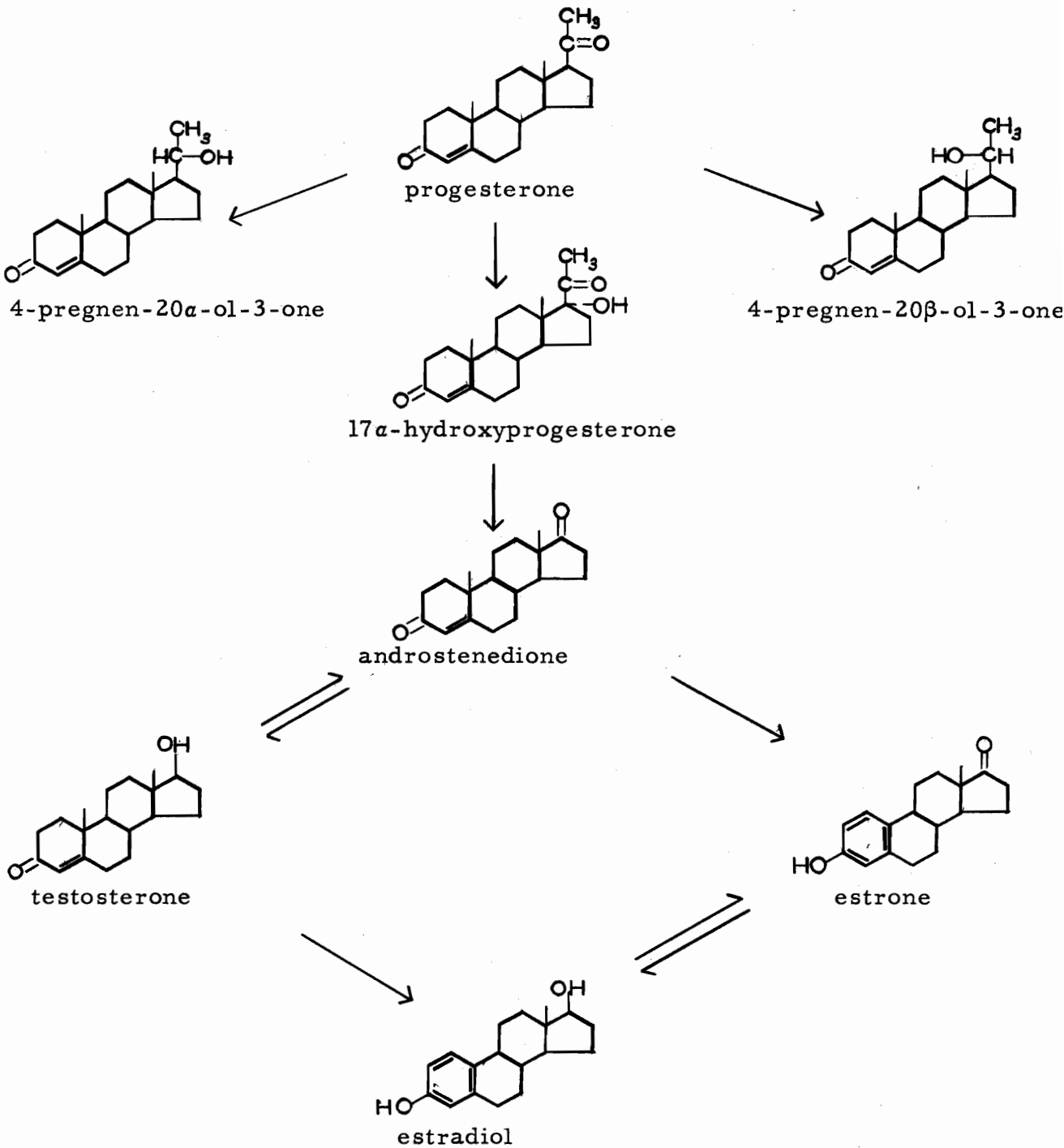


Figure 4. The pathway of estrogen biosynthesis and metabolism in ovarian tissue.

In addition to what appears to be the main pathway of estrogen biosynthesis in ovarian tissue, several workers have found formation of both the 20α- and 20β-hydroxy reduction products of the 20 ketone of progesterone and 17α-hydroxy progesterone (201-204, 194, 196), and two of these compounds, 4-pregnen-20α-ol-3-one and 4-pregnen-20β-ol-3-one, have been isolated from ovarian tissue (205-207).

Biosynthesis of androgenic hormones by testicular tissue has been found to follow the same biosynthetic pathway from pregnenolone or progesterone as described for other steroid producing tissues. In 1951 Brady reported the formation of C^{14}-labeled cholesterol and testosterone after incubation of C^{14}-acetate with testis tissue slices from hog, rabbit, or human (64). The synthesis of testosterone was reported to be stimulated by the in vitro addition of HCG. Savard et al. (208) substantiated Brady's results by demonstrating the formation of testosterone and androstenedione from acetate perfused through testes of a patient with prostatic carcinoma. This conversion was also enhanced by addition of gonadotropin to the perfusate. Using testis tissue homogenates from rats pretreated with HCG, Slaunwhite and Samuels demonstrated the formation of 17α-hydroxyprogesterone and androstenedione from progesterone and presented evidence indicating that carbons 20 and 21 of 17α-hydroxyprogesterone were removed as a two carbon unit in formation of the C-19 product (209). Lynn and Brown found similar reactions to

those reported by Slaunwhite and Samuels and demonstrated the formation of androstenedione from testosterone (210). In a brief communication (211) Lynn reported isolation of 4-pregnen-17α, 20β-diol-3-one and that evidence had been obtained which indicated that this compound was an intermediate between 17α-hydroxyprogesterone and androstenedione. However, in a later paper (212) Lynn and Brown retracted this suggestion and showed, by inhibition of the reductases at pH 8.5 that 17α-hydroxyprogesterone was the immediate precursor of the C-19-steroids, since cleavage of the 17α-hydroxyprogesterone side chain still occurred to form androstenedione. At this same pH, the 17β-reductase was also inhibited and formation of androstenedione from 17α-hydroxyprogesterone was noted in guinea pig testis incubations. Since tissue from this species, as opposed to that of the rat, normally forms primarily testosterone in in vitro incubations, this was direct evidence that androstenedione is the immediate product of the side chain cleaving enzyme, and testosterone is formed by reduction of the 17-ketone of androstenedione. These workers also reported that the 17-hydroxylase and the side chain cleavage enzymes require TPNH and molecular oxygen, while the 17- and 20-ketone reductases require TPNH. The 17, 20-glycol previously reported was identified as the 20β-isomer. In other studies Savard et al. found progesterone metabolized to 17α-hydroxyprogesterone, androstenedione, and testosterone by human neoplastic testicular tissue (213). These results were confirmed by Viscelli and coworkers (214), who also reported the

tentative isolation of the 17, 20-glycol reported by Lynn and Brown in guinea pig, rat and bovine tissue incubations. Savard and Goldzieher extended the androgen biosynthetic data to the stallion in perfusion studies of testes from an animal pretreated with HCG (215). Acetate-1-C^{14} substrate was utilized in the formation of progesterone, 17α-hydroxy-progesterone, androstenedione, and testosterone. Recently, Dominquez et al. have reported the occurrence of 21-hydroxylase in mouse and rat testes (216). With progesterone-4-C^{14} substrate very small amounts of deoxycorticosterone were isolated from incubations with normal tissue and interstitial cell tumors. Forcielli et al. have found an alternate pathway of testosterone biosynthesis in rat testis homogenate (217). Incubation of this tissue with 17α-H^3-progesterone yielded tritiated testosterone, indicating direct formation of testosterone from progesterone without the intervention of 17α-hydroxyprogesterone or androstenedione intermediates, in which case the tritium on carbon 17 would have been lost. From this information the biosynthetic pathways in Figure 5 may be proposed for normal mammalian tissue.

Estrogen biosynthesis in testis tissue can also occur. Wotiz (218) working with embryonic testicular carcinoma, Rabinowitz with human, dog, and cat testes (219), Nyman et al. with perfused stallion testis (220), and Savard et al. (221) with a virilizing testicular tumor have demonstrated the incorporation of C^{14}-acetate into estrone and estradiol. Rabinowitz

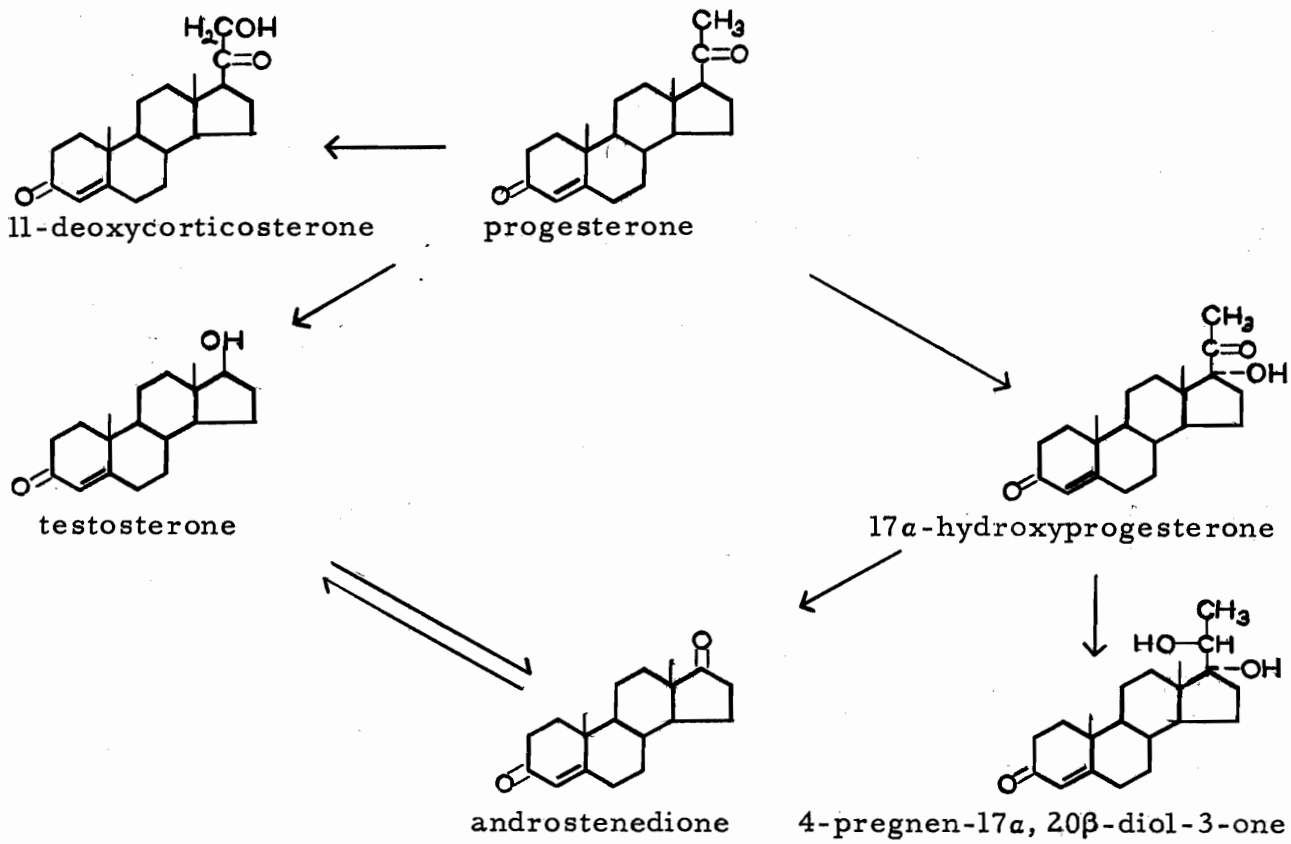


Figure 5. The pathways and progesterone metabolism in normal mammalian testicular tissue.

and Raglund have also reported the ability of testicular tissue from a patient with prostatic carcinoma to synthesize estradiol from C^{14}-mevalonic acid (222), while Baggett and coworkers demonstrated the ability of stallion testes homogenates to convert testosterone to estrogens (184).

Other reactions which may take place in testicular tissue are: 11β-hydroxylation, shown by Savard et al. in interstitial cell tumor tissue with either testosterone (223), or progesterone as substrate (221); and reduction of the 20-ketone of progesterone to both the 20α- and 20β-isomers in normal and embryonal carcinoma testicular tissue from mice (224).

The work to date has elucidated metabolic pathways of steroid hormone biosynthesis in testicular tissue of several species of mammals. Although the presence of androgenic material has been found in both fish (225) and bird (15) testes, no work has been done to elucidate metabolic pathways or to critically identify the hormones produced in any other class of vertebrates than mammals. The closest approach to the chemical identification was a report by Lofts and Marshall (4) on the isolation and partial chromatographic identification of androsterone, androstenedione, progesterone and possibly androsta-1, 4-diene-3, 17-dione from pigeon testis extracts.

The main points to be summarized from this review of previous work is the lack of knowledge of the biochemical aspects of avian

endocrinology. Even though many effects of androgenic hormones in birds are known, such as the chick or capon comb growth, the sparrow beak assay, and the yellowing of the starling's beak, there is no knowledge of the chemical nature of the androgenic hormones produced by the bird. Furthermore, whereas the physiological knowledge of avian testicular cycles is extensive, the information concerning the endocrine function of the testes during these cycles is, at best, indirect.

Since the early investigations on steroid biosynthesis in the late forties, all steroid producing tissues of mammalian origin have been studied with regard to the pathways used to synthesize the hormones elaborated. But with the exception of work concerning the in vitro secretion of adrenal steroids in amphibians, fish, and birds, no studies have been done to elucidate pathways of biosynthesis of steroid hormones in non-mammalian species.

It was with the aim of adding the first spade full of information to this gap in the knowledge of endocrinology that the following studies were performed.

III. EXPERIMENTAL MATERIALS AND PROCEDURES

A. Experimental animals. The English sparrow, Passer domesticus, was selected as the experimental animal for the following reasons: (i) it was easily obtained in large numbers, (ii) it required little space and food, (iii) the amount of testicular tissue provided by each bird was relatively large, (iv) it showed the annual testicular cycle characteristic of many Temperate Zone species of birds (82), and (v) it possesses a secondary sex character that can be used for androgen bioassays (226).

The birds were trapped in the Salt Lake City area in mist nets (227). Animals that were not immediately used were housed in outdoor aviaries under natural climatic conditions. Water and a mixture of equal parts of chick starter mash and millet seed were provided ad lib.

Birds being stimulated experimentally were kept in 18 x 12 x 9 inch breeding cages. Generally two birds were placed in a single cage. Artificial photoperiods were produced in environmental chambers.

B. Chemicals.

1. Human chorionic gonadotropin. Human chorionic gonadotropin (HCG) was obtained as a gift from the Upjohn Company. Five thousand international units (IU) of dry powder was dissolved in 2 ml of sterile water containing 5 mg of chlorobutanol per ml as preservative. A second human chorionic gonadotropin preparation A. P. L., containing 1000 IU per ml

of water with 0.5% phenol, was generously supplied by the Ayerst Company. These HCG preparations were used interchangeably.

2. 4-pregnen-3,20-dione-4-C^{14} (progesterone-4-C^{14}). Lot numbers 16-275A-2 and CFAX-148 progesterone-4-C^{14} were obtained from New England Nuclear Corporation. Specific activities were 20.4 and 16.9 mc/mmole respectively. These were diluted with unlabeled progesterone to obtain solutions of desired specific activity as indicated under the individual experiments.

3. 4-pregnen-3,20-dione-21-C^{14} (progesterone-21-C^{14}). Progesterone 21-C^{14} of 0.71 mc/mmole specific activity was obtained from C. E. Frosst and Company and used without further purification.

4. 4-pregnen-17α-ol-3,20-dione-4-C^{14} (17α-hydroxyprogesterone-4-C^{14}). Lot number 16-83-5, 17α-hydroxyprogesterone-4-C^{14} was purchased from New England Nuclear Corporation. The stated specific activity was 14 mc/mmole. This was diluted with unlabeled 17α-hydroxyprogesterone to obtain the specific activities indicated in the individual experiments.

5. 4-androsten-3,17-dione-4-C^{14} (androstenedione-4-C^{14}). Androstenedione-4-C^{14}, lot number 25-112-14, was used as procured from the New England Nuclear Corporation. The specific activity was 2.0 mc/mmole.

6. 4-androsten-17β-ol-3-one-4-C^{14} (testosterone-4-C^{14}).

Testosterone-4-C^{14} was obtained from Tracerlab Company. Lot number 376-139A-2 had a specific activity of 3.7 mc/mmole and was used without further purification.

All of the commercial C^{14} labeled steroids used as substrates were pure by chromatographic criteria.

7. 4-pregnen-20β-ol-3-one-4-C^{14}. The 4-pregnen-20β-ol-3-one-4-C^{14} used as substrate was isolated from an incubation of avian testicular tissue with progesterone-4-C^{14}. Since it appears that the stimulated avian testis contains little, if any, progesterone or 4-pregnen-20β-ol-3-one (4), the specific activity was assumed to be the same as the progesterone-4-C^{14} substrate, 13.3 mc/mmole. The 4-pregnen-20β-ol-3-one-4-C^{14} was purified in several paper chromatographic systems.

8. Cofactors. The triphosphopyridine nucleotide (TPN), diphosphopyridine nucleotide (DPN), adenosine triphosphate (ATP), and reduced triphosphopyridine nucleotide (TPNH) were obtained, with two exceptions, from the Sigma Chemical Company and used without further purification. In one experiment Nutritional Biochemical Corporation ATP was used and in a second Pabst ATP, lot number 101. All of the Sigma TPN, DPN, and ATP were more than 95% pure. The Sigma TPNH was labeled 87% pure.

Although fumarate and nicotinamide are not actually cofactors, they are included as such since they were handled in a manner identical

to the true cofactors in these studies. Chemical Commerce Company sodium fumarate was utilized as obtained, as was lot number 8265B nicotinamide, procured from General Biochemicals Incorporated.

9. Buffers. Krebs-Ringer phosphate and bicarbonate buffers were prepared as described by Umbreit et al. (228). Serum buffers were made by mixing equal volumes of Krebs-Ringer bicarbonate buffer and bovine or chicken serum. All buffers were adjusted to pH 7.4.

10. Serum. Bovine and chicken blood were collected at local slaughterhouses. Serum was prepared as described by Todd and Sanford (229).

11. Standard Steroid Solutions. Standard steroid solutions were prepared by dissolving weighed quantities of crystalline steroids in vacuum redistilled methanol. These solutions were stored at 4° C. The concentrations of those solutions used to prepare substrates for the incubations were periodically determined by their ultra-violet absorbance at 240 mμ. Only Δ^4-3-keto steroids were used as substrates.

12. Solvents. All solvents were redistilled prior to use. Those used for spectral analyses were also vacuum redistilled.

13. Fixing solutions. Bouin's solution was generously supplied by Dr. K. R. Brizzee's laboratory in the Department of Anatomy, University of Utah, Salt Lake City, Utah. A 10% formalin solution was prepared by diluting Mallinkrodt formalin 1:10 with deionized water.

14. 1, 2-dihydroxypropane (propyleneglycol). USP propyleneglycol was obtained from Braun-Knecht-Heiman Company and used without further purification.

15. Pyridine. Lot number 20605 pyridine from J. T. Baker and Company was vacuum redistilled and stored in a desiccator.

16. Chromium trioxide (CrO_3). Baker and Adamson chromium trioxide was stored in a desiccator and used as obtained.

17. Acetic anhydride. Lot number 92508 acetic anhydride was purchased from J. T. Baker Chemical Company and stored in a desiccator after vacuum distillation.

18. Acetic acid. Baker and Adamson glacial acetic acid was vacuum redistilled over acetic anhydride and stored in a desiccator.

19. Sulfuric acid (H_2SO_4). Dupont reagent grade concentrated sulfuric acid (95-98%) was used to develop sulfuric acid spectra of steroids.

20. Phenol reagent. Folin-Chiocalteu phenol reagent was purchased from the Medical Chemical Corporation. This material was 2.1 N acid by titration and was diluted 1:1 with glass redistilled water before use.

21. Scintillation mixture. The solution in which samples were placed for counting in the Packard Tricarb scintillation counter was prepared by dissolving 4 gm of 2, 5-diphenyloxazole (PPO) and 50 mg of 1, 4-bis-2-(5-phenyloazolyl)-benzene (POPOP) in 1 liter of redistilled

toluene. The solution was stored in a brown bottle at 4° C.

22. Silica gel. Davison and Company silica gel, grade 12 and mesh size 28-200, was purified and activated as described by Sweat (230).

23. Serum albumin. Bovine serum albumin that had been recrystallized four times was obtained from Dr. S. R. Dickman, Department of Biological Chemistry, University of Utah, Salt Lake City, Utah. This material was dried in an oven at 60° C overnight and stored in a vacuum desiccator. Standard solutions, containing 500 μg of albumin per ml, were prepared in glass redistilled water. These solutions were stored in a refrigerator.

24. Chromatography paper. For most of the chromatographic separations, Whatman number 1 filter paper was used. However, if it was desired to perform a spectral analysis of a steroid following paper chromatography, the filter paper was washed prior to use. This was done by hanging the cut strips of paper in a chromatography tank and allowing deionized water to run over and through the paper for 1 week. The strips were then washed similarly in methanol for 1 week. The paper was allowed to dry and stored between two uncut pieces of filter paper.

25. Nitrogen. Bottled nitrogen was purchased from the Whitmore Oxygen Company and filtered through glass-wool prior to use.

C. Equipment.

1. Shaking incubator. A Research Specialties model 2156 constant temperature shaking water bath was used in all but one incubation. In one experiment a Dubnoff Metabolic Incubator from Precision Scientific Company was also employed.

2. Strip counter. Thin window gas-flow automatic strip counters equipped with Brown recorders were used to locate and quantitate radioactivity on paper chromatogram strips (231). These counters were designed and built in the Department of Anatomy, Radiobiology Laboratory, University of Utah, Salt Lake City, Utah.

3. Plate counter. During the early parts of the study the radioactivity in a solution was determined by counting aliquots, applied to aluminum planchets, in a Nuclear Chicago windowless plate counter equipped with an automatic sample changer. This instrument was also used to determine specific activities of steroids.

4. Scintillation counter. More accurate determinations of radioactivity were made in a Packard Tricarb Liquid Scintillation Spectrometer model 314X.

5. Spectrophotometers. Beckman model DK-2 recording, or DU manual, spectrophotometers were used to determine the absorbancy of solutions at various wave lengths. A Perkin-Elmer model 21 double beam recording spectrophotometer was used to record infrared

absorbtion spectra.

7. Haines Scanner. Ultraviolet absorbing material on paper chromatograms was located by use of a scanning device described by Haines (232).

8. Autoclave. A Wilmont-Castle Company model 999 autoclave was used to sterilize glassware at 25 psi and 127° C for 20-30 minutes.

9. Balances. Aluminum planchets and milligram quantities of cofactors, steroids, and serum albumin were weighed on a Mettler model B-6 balance. Gram quantities of chemicals were weighed on a Mettler model H-5 balance. Testes of birds used in the qualitative portion of the studies were weighed on a style 269 pharmaceutical torsion balance produced by the Torsion Balance Company. Testes used in the quantitative studies were weighed on either a 50 or a 500 mg capacity, Roller-Smith Precision Balance.

10. Plating equipment. A revolving planchet holder was placed beneath a hand hair-dryer suspended from a ring stand. A stream of warm air was directed at the planchet holder from the hair dryer. Solutions to be counted were applied to the revolving planchet by means of a micro-pipette. The solvent was evaporated by the stream of warm air from the hair-dryer. This gave a fairly uniform distribution of the solute over the entire area of the planchet.

11. Gas chromatography apparatus. Gas chromatographic analyses

were performed on a Wheelco model 10 "Gas-liquid Chromatograph" equipped with a radium ionization detector. This instrument was made available through the courtesy of Dr. E. C. Horning at the National Institutes of Health, Bethesda, Maryland.

12. pH meters. A Beckman model "H" pH meter was used to measure the pH of buffer solutions, and a model "G" equipped with "one drop electrodes" was used to measure final incubation media pH.

13. Environmental chambers. Two environmental chambers were obtained from the Department of Experimental Biology, University of Utah, Salt Lake City, Utah. These consisted of large, light proof boxes through which fresh air was circulated. Each box was equipped with a 24 hr. timing switch, which permitted regulation of the daily photoperiod within the box. The more elaborate of the two was illuminated by three 40 watt fluorescent light bulbs, held 15, 9 x 12 x 18 inch breeding cages, and could be kept at a constant temperature. The other was illuminated by six 25 watt incandescent lamps, held 6 breeding cages, and operated at room temperature.

D. Procedures.

1. Animal stimulation. Because of the annual cycle in the size of sparrow testes, the period of the year when a large enough quantity of testicular tissue could be obtained from one bird was limited to the spring and early summer (93). At this time the testicular weight was

greater than 100 mg (Figure 1).

In late summer and early fall the testes are normally regressed following the breeding season, and some part of the hypothalamic-pituitary system apparently is not responsive to stimulation by increased photoperiods (118). Daily injection of HCG during this period produced enough testicular tissue with which experiments could be carried out. Table I shows a preliminary experiment in which a dosage of HCG was determined for future injections. From these results it appeared that 500 IU daily for 2 weeks was the maximum that could be tolerated by the birds. This dosage was used in succeeding experiments unless the animals appeared to be too sick during the latter stages of the injection period. In such a case, the daily dosage was reduced to 250 IU daily for the final four days. By mid-November the birds became responsive to increased photoperiods. They were then placed in breeding cages in an environmental chamber and subjected to eighteen hour daily photoperiods. For the qualitative studies the animals were thus stimulated for a minimum of 2 weeks, which usually produced a combined testicular weight in excess of 100 mg. Birds used in the quantitative studies were stimulated for 5-7 weeks and were generally found to have combined testicular weights between 200 and 400 mg. This latter treatment was considered to effect maximal stimulation of the gonads.

In an attempt to obtain birds that were physiologically mature, or

Table I. Determination of the tolerance and response of English sparrows (Passer domesticus) to human chorionic gonadotropin (HCG).

Bird	Daily HCG Dose IU	Injection Date Initial	Injection Date Final	Final Testicular Weight, mg.
19	500	11-24	12-8	180.8
18	500	"	11-29	12.4
09	500	"	12-2**	52.1
08	1000	"	11-30	19.3
10	1000	"	11-28***	11.1
11	1000	"	11-29+	52.0
12	1500	"	11-26‡	6.5
13	1500	"	11-27Δ	8.3
14	1500	"	11-28***	10.6
15	0*	"	11-29	3.8
16	0	"	12-2	5.2
17	0	"	12-8	3.2

* Controls injected with sterile water

** Found dead on 12-3

*** Found dead on 11-29

\+ Found dead on 11-30

‡ Found dead on 11-27

Δ Found dead on 11-28

whose gonads had produced androgens and sperm, but were in an inactive state at the time of the experiment, male sparrows were trapped in late November and December. These birds were stimulated with an 18 hr. daily photoperiod for a minimum of 2 weeks, or until testicular stimulation was evident by a blackening of the beak. They were then placed in outdoor aviaries under natural photoperiods and the testes allowed to regress 1-2 months.

2. Tissue preparation. Birds used for an incubation were placed in a walk-in cold room and sacrificed by decapitation. The testes were immediately removed, freed from adhering connective tissue, and weighed. After weighing the tissue the specimens to be preserved for histological examination were placed into either a 10% formalin or Bouin's solution. Testes to be used in the incubation were placed at once into ice-cold buffer 80 mM in nicotinamide. When the necessary amount of tissue had been obtained, so the buffer to tissue ratio was ten to one (v/w), the tissue was homogenized at 0° C in a Ten-Broeck glass tissue grinder for three to four minutes until no large tissue particles were visible. The homogenate was centrifuged at 700 x g for ten minutes at 4° C; the supernatant decanted and placed in an ice bath. This same procedure was used when pancreas or muscle tissue was used as tissue blanks.

In the experiments where whole homogenates were used, the

centrifugation step was omitted. When tissue slices were employed the testes were cut into slices by hand and the slices placed directly into incubation flasks containing buffer. Since tissue slices were used only in the preliminary investigations, a more uniform tissue slicing method, such as that attained with a Stadie tissue slicer, was never employed.

3. Preparation of incubation flasks. Because microbiological transformations of steroid molecules are well known (233), all glassware and surgical instruments used in an incubation were sterilized in an autoclave prior to use. Glass stoppered 125 ml or rubber stoppered 25 ml Erlenmeyer flasks were used as incubation vessels in all experiments. Substrates labeled with C^{14} were added to the flasks in 0.2 ml of propyleneglycol:methanol (1/1), and the methanol evaporated at 41° C under a stream of nitrogen. The following description pertains to incubations in which 125 ml Erlenmeyer flasks were used. When the 25 ml Erlenmeyer flasks were used all volumes were divided by four.

Four ml of buffer containing nicotinamide, 5 ml of buffer containing all other cofactors, and 1 ml of homogenate supernatant were added to the incubation flasks in that order. The final cofactor concentrations, unless otherwise noted in the results, were: (i) 40 mM nicotinamide, (ii) 4 mM DPN, ATP, TPN or TPNH, and (iii) 1 mM sodium fumarate. Nicotinamide, TPN, and sodium fumarate were the cofactors routinely included. Each flask was gassed with 95% O_2-5% CO_2 for 2 minutes immediately after addition of the homogenate and tightly stoppered.

4. Incubation. The flasks were then placed in the shaking incubator at 41° C (234). In general a 3 hour incubation period was used for the qualitative portions of the study, and 0, 15, and 30 minute periods were used for the quantitative investigations. Two "blank" flasks were included in the 3 hour incubations. These were treated identically to the other incubation flasks except that no tissue was added. In the zero time controls of the quantitative studies, the reaction was terminated immediately after the 2 minute gassing period. At the end of the incubation period the reactions were stopped by adding 10 ml of diethyl-ether:chloroform (4/1, v/v) to the incubation medium and mixing thoroughly. In several incubations the pH of the incubation medium was determined just prior to stopping the reactions, and was found to be about 7.1.

5. Extraction. The 10 ml aqueous medium was extracted five times with equal volumes of the diethylether:chloroform solvent directly after termination of the incubation. When the total incubation volume was 2.5 ml, the medium was diluted to 10 ml with water prior to extraction. Mixing of the aqueous and organic phases to achieve thorough extraction was accomplished with "footed" glass stirring rods (235). If an emulsion formed, it was separated by centrifugation for 10-15 minutes at 2000 rev/minute. The five volumes of organic extract were combined and evaporated to dryness at 40° C under a stream of nitrogen. At this

time, in the majority of the experiments, 50 or 100 μg of both testosterone and androstenedione were added to each sample. In a few of the quantitative studies 4-pregnen-20α-ol-3-one was used instead of androstenedione. These added compounds served as reference points for the ensuing chromatographic separations and purifications.

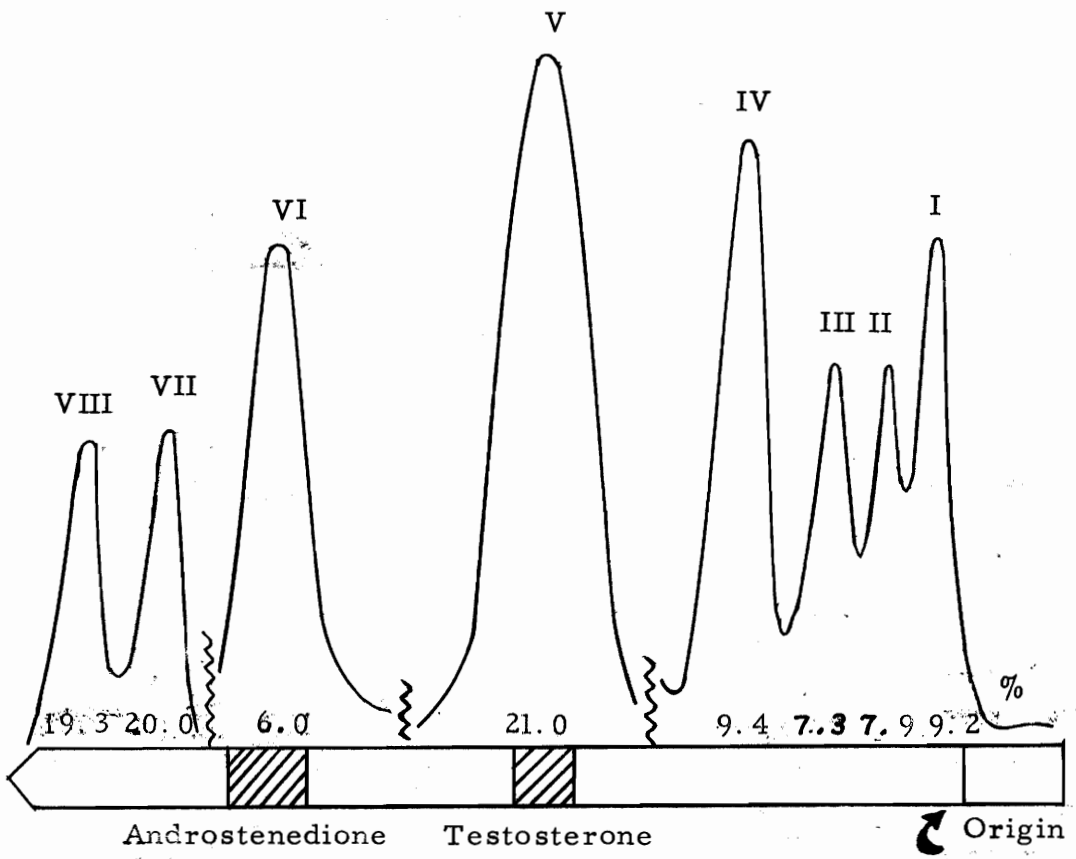
The per cent recovery of metabolized substrate for the qualitative studies was calculated on the basis of the substrate recovered from the blank incubation flasks, which had no tissue added. The dpm calculated for peaks in the chromatogram (cf. p. 62) were summed, and this figure divided by the average dpm in the substrate peak from the two blank incubation flask chromatograms. Calculated in this manner recoveries of metabolized substrate averaged between 95 and 100%, with a range of 90-110%.

Extraction recoveries in the qualitative studies were calculated by determining the radioactivity remaining in the aqueous medium by counting an aliquot in the scintillation counter. A sample of the original substrate solution was counted at the same time. Using this figure as 100%, the per cent remaining in the aqueous phase after extraction was calculated. This figure was subtracted from 100 to give a per cent recovery figure. An average of 18 such determinations showed a recovery of 99.9%, with a range of 99.7 to 100%.

6. Purification, isolation, and identification.

(a) First standard chromatographic separation. The samples were applied to 2 or 3 cm paper strips by means of capillary pipettes (236) and chromatographed in the hexane/formamide system (237). The chromatograms were allowed to run for 1 hour after the mobile phase had reached the end of the strip, and the droppings from each strip were collected. The chromatogram was removed from the hexane/formamide tank; the hexane permitted to evaporate from the paper; and the strips developed in hexane:benzene (1/1)/formamide. When the mobile phase reached the end of the strips, the chromatogram was removed and dried in a hood overnight. Aliquots of the droppings from the original chromatogram of several incubations were plated on aluminum planchets and counted on a plate counter to check the possibility of C^{14} containing metabolites less polar than progesterone being formed.

Ultraviolet absorbing compounds, the Δ^4-3-ketosteroids added to the samples, were located on the dry chromatogram by means of a Haines scanner. The strips were then counted for radioactivity on a strip counter. Using progesterone-4-C^{14} as substrate, a typical tracing obtained by this method from an incubation with avian testicular tissue is shown in Figure 6. Areas of the chromatogram containing radioactive metabolites were eluted with methanol. Wherever resolution of adjacent metabolites on the chromatogram was not attained, the



I - incompletely identified
II - incompletely identified
III - 4-pregnen-17α, 20α-diol-3-one
IV - 4-pregnen-17α, 20β-diol-3-one
V - Testosterone and 17α-hydroxyprogesterone
VI - 4-pregnen-20α-ol-3-one and (androstenedione)
VII - 4-pregnen-20β-ol-3-one
VIII - progesterone

Figure 6. Tracing of the first chromatographic separation of radioactive metabolites isolated from a typical incubation of avian testicular tissue cell-free homogenate with 4-C^{14}-progesterone substrate. Figures at base of curves are per cent of added substrate.

metabolites were eluted together and separated on subsequent chromatograms.

(b) Elution techniques. Elution of compounds from paper strips was accomplished by impaling one end of the strip on a 22 gauge hypodermic needle bent in a semicircle at the tip. The hypodermic needle was placed on a 10 ml syringe barrel supported vertically from a ring stand. Methanol was placed in the syringe and allowed to run out slowly through the needle over the paper strip. The solvent drops were collected in a test tube at the end of the paper strip. Between 5 and 15 ml of methanol were used depending on the amount of steroid and length of the strip to be eluted. This gave 90-100% elution of material on the strip as determined by recounting the eluted strip.

(c) Further paper chromatographic purification. After elution from the first chromatogram, metabolites were separated and purified further by paper chromatography. The formamide systems of Zaffaroni (237) were most frequently employed. For identification purposes the propyleneglycol (237) and Bush systems (238) were also used.

(d) Acetylation. When complete separation of two metabolic products by paper chromatography was difficult to achieve, the combined metabolites were acetylated in a 4/1 mixture of pyridine and acetic anhydride by the following procedure. The mixture of compounds was concentrated in the tip of a conical centrifuge tube by methanol rinses

and completely dried under a stream of nitrogen at 40° C. Two hundred microliters of a solution of acetic anhydride in pyridine were added; the tube tightly stoppered and placed in a hood overnight (8-16 hr). At this point two different methods were used, both giving identical results. In the shorter of the two the reaction was stopped by adding 2 ml of methanol. The methanol, pyridine, and acetic anhydride were evaporated to dryness at 40° C under nitrogen, and the residue concentrated in the tip of the tube with methanol rinses. In the longer method the reaction was stopped by addition of 4 ml of water. The aqueous solution was then extracted five times with 5 ml of ethylacetate, the extractions combined evaporated to dryness, and the residue concentrated in the tip of the tube as before. Ninety-five to 100% acetylation of those steroid hydroxyl groups forming acetate esters was achieved under these conditions.

The acetate obtained in either case was applied to paper strips and the chromatogram developed in an appropriate system. Chromatographic mobilities of the acetylated compounds were determined in at least two different paper chromatographic systems. By this method any two products that could not be separated through chromatography of the original compounds were resolved.

Acetylation was also used for identification of metabolites. Complete acetylation of all the steroid hydroxyl groups encountered in these experiments, except the 17α hydroxyl in 21 carbon molecules, was achieved

with the 4:1 (v/v) mixture of pyridine and acetic anhydride. In an attempt to determine the number and possible location of acetylable hydroxyl groups in some of the incubation products, a ratio of pyridine to acetic anhydride of 15:1 was used. Hydroxyl groups at positions 6, 20, and 3 in the steroid molecule have been shown to be incompletely acetylated under these conditions (239). Therefore, when a dihydroxy compound with hydroxyl groups in two of these positions is acetylated with a 15:1 pyridine:acetic anhydride solution, three products are formed, the diacetate and two monoacetates. On the other hand, acetylation of this same dihydroxy compound with a 4:1 ratio of pyridine to acetic anhydride will give only one product, the diacetate. This hypothetical compound could easily be distinguished from another dihydroxy steroid in which one hydroxyl was the same and the second at a different position, such as 21, that is acetylated completely by a 15:1 pyridine to acetic anhydride solution. This second compound would yield only two products, the diacetate and the 21 monoacetate, when acetylated with the dilute acetic anhydride solution.

(e) Saponification. When the amount of metabolite available was small, the acetate derivative was saponified to regain the original compound. Saponification was achieved by dissolving the acetate in 0.5 ml ethanol to which 0.5 ml 2.5% Na_2CO_3 was added. The reaction was allowed to proceed at room temperature overnight. At the end of

the reaction period the ethanol was evaporated; the solution diluted to 5 ml with water; and the saponified material and remaining ester extracted with five equal volumes of ethylacetate. The free and esterified compounds were separated by paper chromatography. If the steroid ester was difficult to saponify by this means, the acetylated compound was dissolved in 1 ml of 0.1N ehtanolic sodium hyrdoxide and incubated at 37° C for 30 minutes. The solution was then diluted with 5 ml of water, the ethanol evaporated, and the steroid extracted and chromatographed as previously described.

A situation in which these two methods could be compared was the saponification of the acetate derivative of 4-pregnen-20α-ol-3-one. The Na_2CO_3 method consistently saponified about 20% of the ester while the NaOH method hydrolyzed about 50%.

(f) Oxidation. The oxidation of purified metabolites was accomplished by use of a saturated solution of chromium trioxide (CrO_3) in glacial acetic acid. A few crystals of CrO_3 were placed in a glass-stoppered tube. Approximately 0.5 ml of glacial acetic acid was added to the tube; the mixture shaken for several minutes; and the liquid phase removed with a Pasteur pipette and discarded. Fresh acetic acid was added and the procedure repeated twice to remove any water in the CrO_3. Finally, a volume of acetic acid sufficient to provide enough solution for the number of samples being oxidized was

added to the remaining CrO_3 and shaken until saturation of the liquid was achieved. Two hundred microliters of the saturated solution were added to each sample in a test tube, and the tube stoppered. After 30 min., 5ml of water were added to the tube, and the aqueous solution extracted immediately with 5 ml of ethylacetate. Extraction was repeated four times; the extraction volumes were combined and evaporated to dryness. The extracted oxidized steroid was concentrated in the tip of a conical centrifuged tube, applied to 2 cm paper strips, and chromatographed in an appropriate system. By this method 80-100% of testosterone was oxidized to androstenedione and about 60% of either 4-pregnen-20β-ol-3-one or 4-pregnen-20α-ol-3-one to progesterone.

(g) Sulfuric acid spectra. Two ml of sulfuric acid were added to a test tube containing the dry sample. It was placed in the dark at room temperature and allowed to develop for two hours. At the end of this period the absorption spectrum was read against a sulfuric acid blank on the DK-2 spectrophotometer. This is essentially the method described by Zaffaroni (240).

(h) Preparation for IR analysis. Metabolites prepared for micro-infrared analyses were extensively purified by paper chromatography. After elution of the final paper chromatogram, the steroid was further purified on a micro-silica gel column as described by Sweat (230). Vacuum redistilled solvents were used for preparation and

elution of the column.

(i) Gas chromatography. Retention times of metabolites and authentic compounds were obtained by gas chromatography after paper chromatographic purification. A 6 ft. x 4 mm column packed with gas-chrom P as solid support was used for all determinations. The liquid phase was 3/4% silicone rubber gum and argon the carrier gas. Columns were operated at 200° C and a gas pressure of 14 psi. A potential of 750 volts was used in the ionization detector. Retention times relative to a cholestane standard were calculated for each determination.

(j) Crystallization techniques. In order to recrystallize an isolated and purified C^{14}-metabolite to constant specific activity, 10-20 mg of authentic, crystalline steroid were added to the radioactive metabolite. This mixture was dissolved in 1-2 ml of methanol and 100 µl aliquots applied to preweighed aluminum planchets. The remaining methanolic solution was evaporated to dryness. The residue was redissolved in a minimum of hot solvent, or a mixture of two solvents, from which the steroid was to be crystallized. The solution was chilled on ice. If the steroid did not crystallize immediately, either some of the solvent was evaporated, or more of the component of the solvent mixture in which the steroid was less soluble was added to the solution. Following crystallization the sample was centrifuged in a refrigerated centrifuge, if necessary, and the supernatant removed and discarded.

The crystalline material was dissolved in from 0.8 to 1.8 ml of methanol and duplicate 100 µl aliquots applied to aluminum planchets as before. The volume of methanol used was that which would produce an approximate concentration of 1 mg/0.1 ml. The crystallization process was repeated two or three times. Different solvent systems were used whenever possible. The planchets were stored in a vacuum desiccator overnight, reweighed, and counted on a plate counter.

7. Elucidation of metabolic pathways. To elucidate the metabolic pathways involved in progesterone metabolism by avian testicular tissue, a relatively large amount, 1 µmole, of unlabeled steroid suspected of being a possible intermediate was added to the incubation medium. If this unlabeled steroid was an intermediate in a biosynthetic sequence of reactions, if would either accumulate radioactivity to a greater extent than in the incubation without the unlabeled steroid "trap", or cause radioactivity to increase in the immediate precursor to the "trap", or both. The C^{14} in the products formed from the "trap" material would be decreased by dilution. This method was used to elucidate the pathways of steroid metabolism with progesterone-4-C^{14} or 17α-hydroxyprogesterone-4-C^{14} as substrates.

In addition, the use of different substrates such as testosterone-4-C^{14}, androstenedione-4-C^{14}, and 4-pregnen-20β-ol-3-one-4-C^{14} was also employed to help clarify the source of isolated metabolites.

8. Quantitation.

(a) Radioactivity. A strip counter was used to detect and quantitate radioactive metabolites on paper chromatograms. Two different counters of the same make were used during these studies. The first was used for the qualitative or semi-quantitative portions of the studies, in which metabolites were identified and metabolic pathways elucidated, and will be referred to as "strip counter 1." The second was used for the quantitative portions of the study in which the enzyme content of the testis tissue was determined and will be referred to as "strip counter 2."

Strip counter 1 was standardized against a windowless plate counter. Duplicate aliquots of a progesterone-21-C^{14} solution were applied to aluminum planchets, and identical aliquots chromatographed in the hexane/formamide system. After developing the chromatogram, it was dried and counted. The aluminum planchets were also counted. This procedure was repeated using various amounts of radioactivity so that all scales of the strip counter could be used. The area under the peak representing the progesterone-21-C^{14} recorded by the strip counter was calculated in square centimeters by triangulation. The counts per minute recorded for the duplicate aliquots applied to the planchet were divided by this area. This gave a figure which was used as a factor to convert area to cpm on the plate counter. As mentioned, this calculation

was performed in duplicate for each scale normally used on this strip counter.

Strip counter 2 was standardized against the Tricarb Spectrometer. Graded amounts of progesterone-21-C^{14} were applied to paper strips in duplicate and chromatographed. After the developed chromatograms had been counted, the progesterone areas were eluted with methanol into individual glass counting vials. The eluted strips were recounted. If the elution had not quantitatively removed all of the C^{14} from the strip, it was eluted again into the same vial. This was repeated, if necessary, until no radioactivity could be detected on the strip. The methanol was evaporated, and 10 ml of scintillation counting fluid added. These samples were then counted in the Tricarb Spectrometer along with the Tricarb blank and standard. The dpm contained in each sample was determined after calculating the counting efficiency by use of the standard. A factor to convert the area recorded from the original chromatogram to dpm was obtained by dividing the dpm of the samples by the area in square centimeters.

Metabolites obtained from incubations were quantitated after chromatographic isolation by calculating the area beneath the appropriate peak corresponding to a radioactive spot on the chromatogram and multiplying this area by the factor determined for that particular scale and counter. In the semi-quantitative studies the average cpm, if any,

on the chromatograms of the two "blank" incubation flasks, in the area corresponding to that being quantitated, was calculated. This figure was substracted from the cpm of the sample to give a "net cpm." This "net cpm" was divided by the cpm in the peak on the "blank" chromatogram corresponding to the substrate used. Multiplying by 100 gave per cent conversion of substrate to metabolite by the tissue added, since no tissue had been added to the "blank" flasks. Millimicromoles of product formed were calculated by assuming 100% to be equal to the number of millimicromoles added as substrate. These methods of calculation automatically corrected for recoveries in the incubation, extraction, and chromatographic procedures.

In the quantitative portions of the studies, an aliquot of non-incubated substrate solution was chromatographed in parallel with the first chromatogram of the extracts of the incubation media. The dpm of this substrate standard was assumed to be equal to the amount of radioactivity used as substrate. The accuracy of this assumption was checked in one incubation by counting a similar aliquot of the substrate solution in the Tricarb scintillation counter and the two figures obtained agreed within 5%. Quantitation of the radioactive metabolites was done by substracting the average dpm of the zero time controls from the dpm in the incubated samples. The resulting figure was referred to as the "net dpm." The net dpm was divided by the dpm added as substrate, as

determined from the standard substrate chromatogram, and multiplied by 100 to yield a per cent conversion figure. The conversion in millimicromoles was calculated by dividing the per cent conversion by 100 and multiplying by the mμmoles of substrate used - 200 in the 10 ml incubations and 50 in the 2.5 ml incubations.

By these methods any radioactive peak on the chromatograms of much less than 400 or 500 dpm, depending on the length of the strip over which it was spread, would not be detected. This would be less than 0.15% of the substrate commonly used in these studies.

(b) Non-radioactive steroids. When large amounts of non-radioactive Δ^4-3-ketosteroid were used as substrate or as traps, it was necessary to follow the conversion of these compounds by their 240 mμ absorption. In order to obtain a fairly accurate and sensitive quantitative measurement, the isolated material was chromatographed on washed paper prior to the measurement of the 240 mμ absorption. A blank strip was included in this chromatogram. After this final paper chromatography, the area of the paper strip containing the steroid to be determined and an identical area of the blank strip were each eluted in 5 ml of vacuum redistilled methanol. The methanol was evaporated to dryness, and both the sample and blank were redissolved in an appropriate amount of vacuum redistilled methanol. The volume used depended on the amount of steroid estimated to be in the sample from the intensity of its ultra-violet absorption on

the paper chromatogram. The sample absorbance was then read against the blank in DK-2 spectrophotometer. The steroid concentration was calculated using the molecular extinction coefficient of the compound being determined. The limit of detection by this method was approximately 1 μgm/ml.

(c) Protein. Protein determinations were performed using the Lowry modification of the Folin-phenol method (241). Glass distilled water was used throughout the determination. A 50:1 dilution of the original cell free homogenate was used to determine the protein content of the homogenate. A standard protein solution prepared from recrystallized bovine serum albumin was assayed at the same time that each sample was analyzed. A typical standard curve obtained by this method is shown in Figure 7.

(d) Enzyme content. The enzyme assay incubations, or quantitative studies, were performed with a progesterone-4-C^{14} concentration of 0.2 μmoles in 10 ml of medium. Reactions were stopped at 0, 15, and 30 minutes. Figures 8-10 show the mμmoles of product formed with respect to time. It invariably appeared that there was a lag period of 5-10 minutes in the 20 reductase activities and the 17α-hydroxylase activity tended to decrease slightly during the 15-30 minute interval. Therefore, the 0-15 minute interval was used to obtain the rate of 17α-hydroxylation, and the 15-30 minute interval used to obtain the rate of

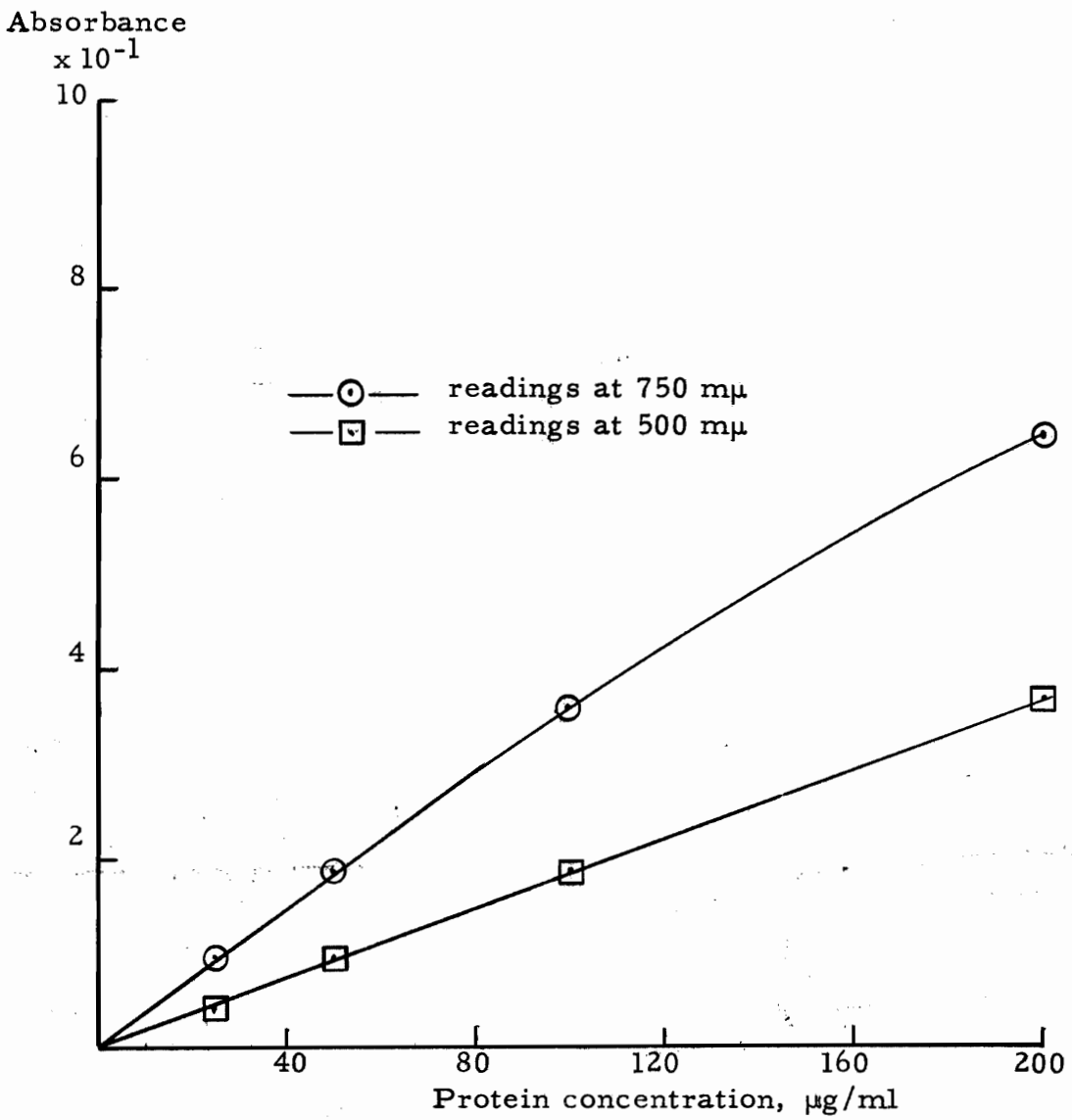


Figure 7. Standard protein determination curve.
Protein: bovine serum albumin.

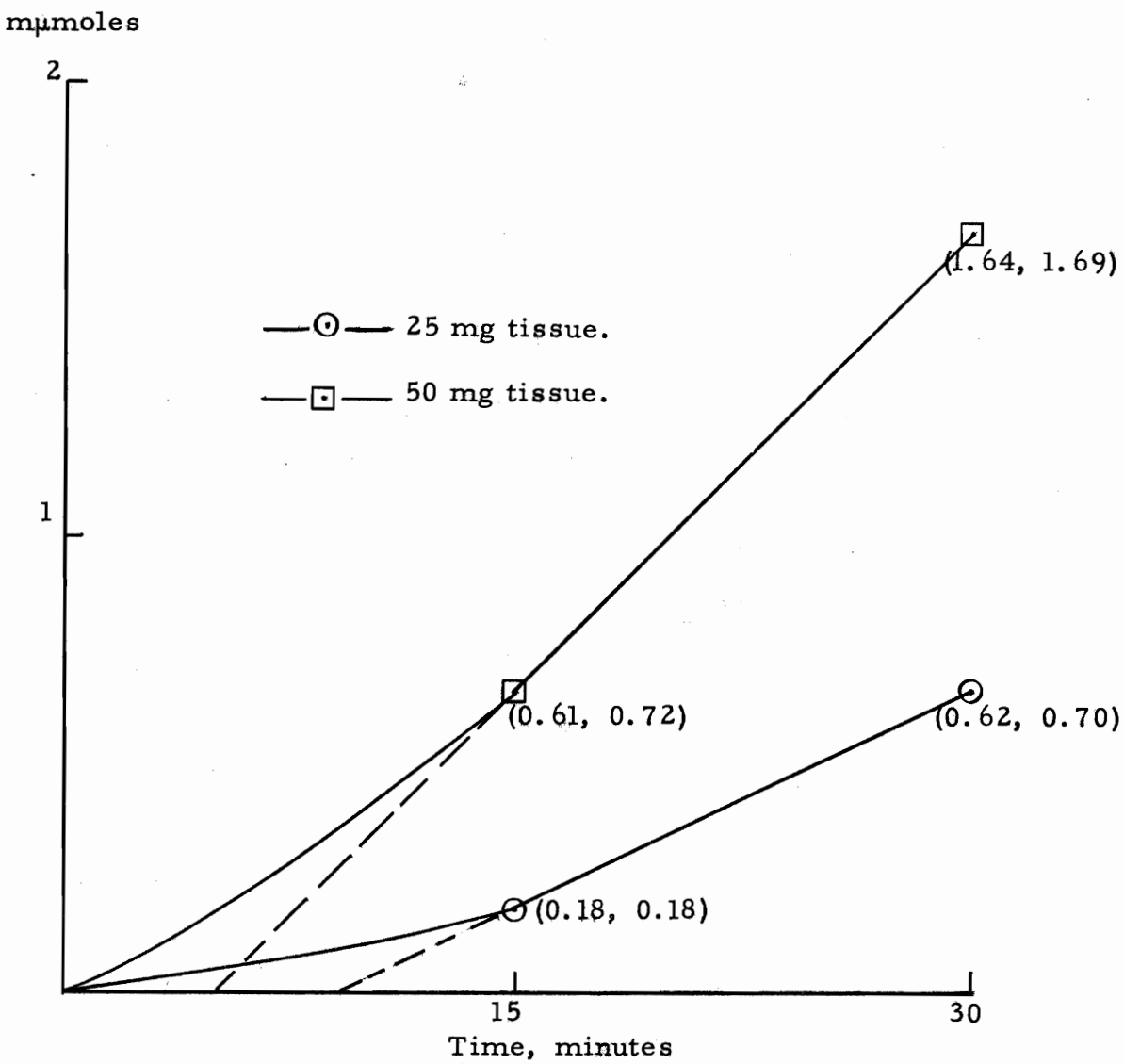


Figure 8. Rate of formation of 4-pregnen-20α-ol-3-one at two tissue concentrations. Testes: birds stimulated Dec. 21 - Feb. 10 with an 18 hr. daily photoperiod.
Average testicular weight: 253.5 mg.
Substrate: 0.05 μmoles progesterone-4-C^{14}; 4. mc/mmole
Final incubation volume: 2.5 ml.
Values in brackets are duplicate determinations.

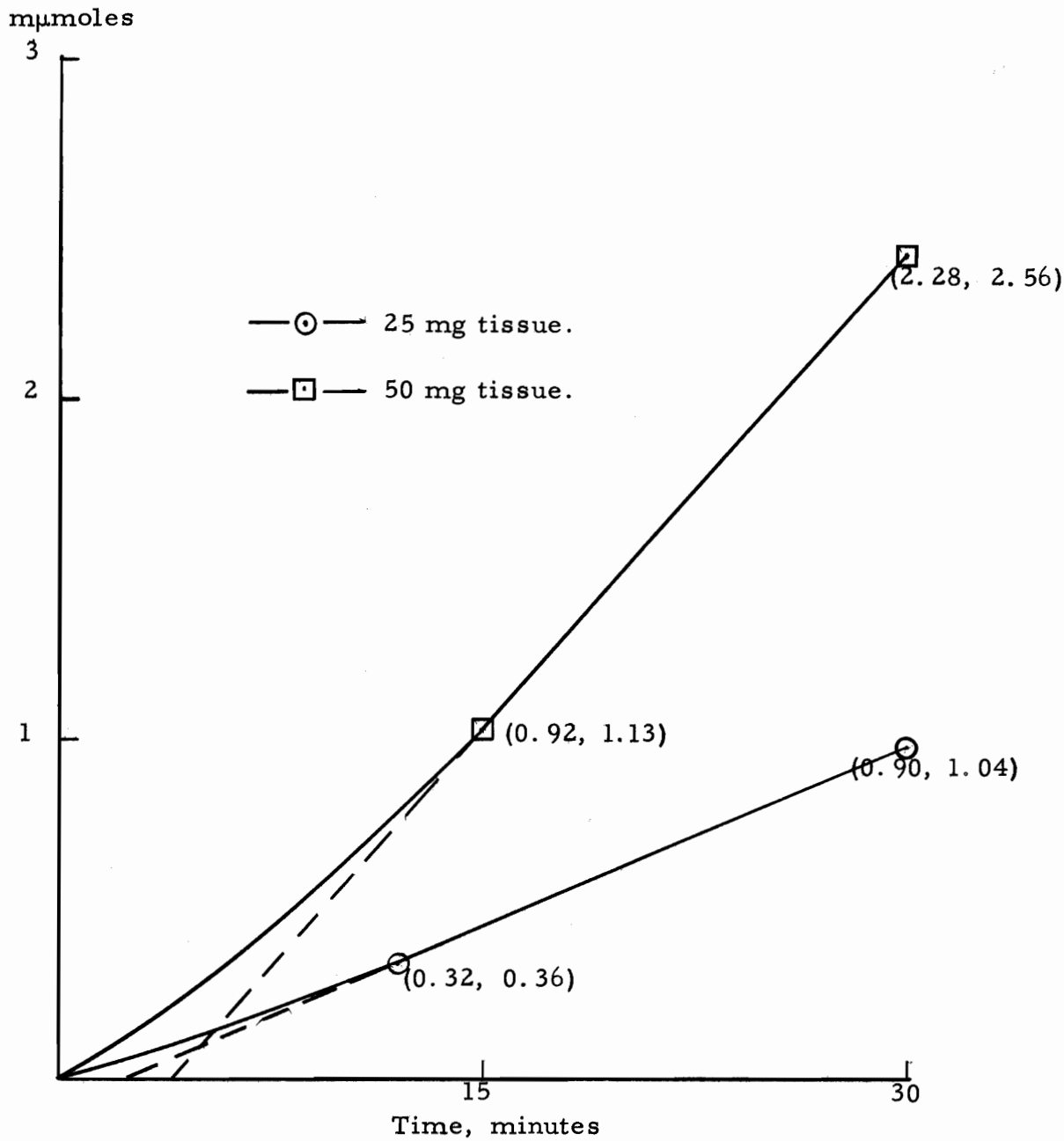


Figure 9. Rate of formation of 4-pregnen-20β-ol-3-one at two tissue concentrations. Testes from birds stimulated Dec. 21-Feb. 10 with an 18 hr. daily photoperiod.
Average testicular weight: 253.5 mg.
Substrate: 0.05 μmoles progesterone-4-C^{14}; 4 mc/mmole.
Final incubation volume: 2.5 ml.
Values in brackets are duplicate determinations.

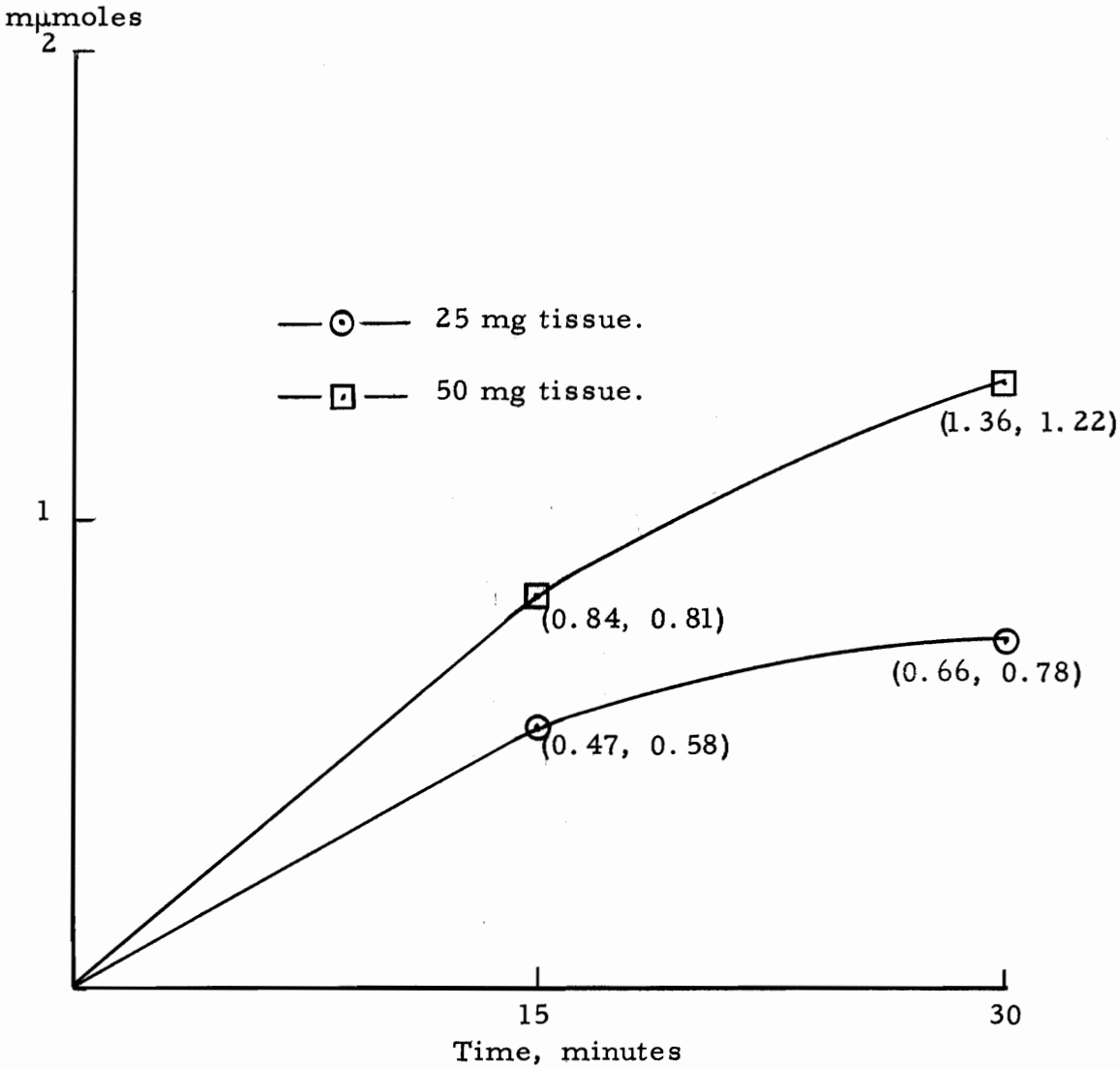


Figure 10. Rate of 17α-hydroxylation at two tissue concentrations.
Testes from birds stimulated Dec. 21-Feb. 10 with an 18 hr. daily photoperiod.
Average testicular weight: 253.5 mg.
Substrate: 0.05 μmoles progesterone-4-C^{14}; 4 mc/mmole
Final incubation volume: 2.5 ml
Values in brackets are duplicate determinations.

20α and 20β reduction. Since the amount of androstenedione formed was insignificant the 17α-hydroxylase activity was measured by the sum of testosterone and 17α-hydroxyprogesterone. The 20α and 20β-reductase activities were measured by the amounts of 4-pregnen-20α-ol-3-one and 4-pregnen-20β-ol-3-one formed, respectively.

To see if these measurements were proportional to the amount of enzyme present, an incubation was done using twice the volume of homogenate usually employed. Thus, the enzymatic activities at two tissue concentrations were compared. These results calculated on a mg of protein basis are shown in Table II.

9. Bio-assay. Authentic compounds corresponding to the metabolites identified from the incubations were qualitatively bioassayed by the method of Pfeiffer (226). Sexually inactive male, or female English sparrows were used as the test animals. Pasteur pipettes were calibrated to determine the delivery of ethanol in drops per ml. Steroid solutions containing the desired daily dose per drop of ethanol were prepared. The pipette calibrated for one steroid solution was used to apply only that particular solution to the beaks of the appropriate test animals. Testosterone was used routinely as a standard and progesterone as the control.

10. Histological preparations. After fixation of tissue all histological preparations were performed by Mrs. D. L. Lerdahl of Dr.

Table II. Comparison of the enzyme activities at two concentrations of pooled testicular tissue.
Testes from birds stimulated Dec. 21-Feb. 10 with an 18 hr daily photoperiod.
Average testicular weight: 235.5 mg.
Substrate: 0.05 μmoles progesterone-4-C^{14}; 4 mc/mmole
Final incubation volume: 2.5 ml.

Tissue Used, mg	Enzyme Activities*		
	17α-OH-ase	20β-red.ase	20α-red.ase
25	1.47	1.78	1.36
50	1.18	1.73	1.42

* Expressed as mμmoles of product formed per mg protein per hour.

Kenneth Brizzee's laboratory in the Department of Anatomy. Serial tissue slices 10μ thick were stained with hematoxylin and eosin and examined under a light microscope.

IV. RESULTS

A. Comparison of tissue preparations. The initial incubations were performed using testis tissue slices. With the object in mind of obtaining a simpler and better defined system, the gross results obtained with slices and a whole homogenate were compared. The average per cent conversion to the three major metabolites for slices and whole homogenate is shown in Table III. Unless otherwise stated all numerical results are averages of duplicate incubations. The degree of metabolism appeared the same or greater in the homogenate as in the slices. For this reason the use of slices was discontinued.

The progesterone metabolism by the whole homogenate was also compared with that of the cell free homogenate. These results are shown in Table IV. There were no significant differences in per cent conversion to any of the major metabolites. Since the more uncomplicated system was still desired, the cell free homogenate was used in all subsequent experiments.

To answer the question of whether the metabolism observed was specific for the testis, two other sparrow tissue homogenates were incubated in parallel with testis tissue. The results in Table V show that pancreas and skeletal muscle metabolized progesterone to a very insignificant extent, if at all, when compared to testis under these conditions.

Table III. Comparison of progesterone metabolism by testis tissue slices and homogenate.
Testes from birds stimulated October 1 - October 31 with an 18 hour daily photoperiod.
Average testicular weight: 65.3 mg.
Substrate: 0.1 μmole progesterone-4-C^{14}; 1.0 mc/mmole.
Final incubation volume: 10 ml.

Tissue Preparation	Average Conversion (%) Metabolites		
	V + V-Ac	VI + VI-Ac	VII
Slices	1.7	1.2	16.5
Whole homogenates	1.4	2.3	29.3

V= 17α-hydroxyprogesterone
V-Ac= Testosterone
VI= Androstenedione
VI-Ac= 4-pregnen-20α-ol-3-one
VII= 4-pregnen-20β-ol-3-one

Table IV. Comparison of progesterone metabolism by cell-free and whole homogenates of pooled testis tissue.
Testes from birds stimulated January 5-22 with an 18 hour daily photoperiod.
Average testicular weight: 133.8 mg.
Substrate: 0.1 µmole progesterone-4-C^{14}; 1.0 mc/mmole.
Final incubation volume: 10 ml.

Tissue Homogenate Preparation	Average Conversion (%) Metabolites				
	V	V-Ac	VI	VI-Ac	VII
Whole	2.5	19.2	0.21	0.47	4.9
Cell-free	2.0	19.6	0.17	0.30	4.6

V= 17α-hydroxyprogesterone
V-Ac= Testosterone
VI= Androstenedione
VI-Ac= 4-pregnen-20α-ol-3-one
VII= 4-pregnen-20β-ol-3-one

Table V. Comparison of progesterone metabolism by testis tissue and two other avian tissue whole homogenates.
Testes from birds stimulated Nov. 16-Dec. 10 with an 18 hour daily photoperiod.
Average testicular weight: 317 mg.
Substrate: 0.05 μmoles progesterone-4-C^{14}; 2.0 mc/mmole.
Final incubation volume: 10 ml.

Tissue Homogenate Preparation	Average Conversion (%) Metabolites		
	V + V-Ac	VI + VI-Ac	VII
Testis	2.8	5.6	29.8
Pancreas	0.3	0*	0.9
Muscle	0*	0*	0*

* below limits of detection.
V=17α-hydroxyprogesterone
V-Ac=Testosterone
VI=Androstenedione
VI-Ac=4-pregnen-20α-ol-3-one
VII=4-pregnen-20β-ol-3-one

Evidence for the enzymic nature of the chemical transformations occurring in the incubations is presented in Table VI. In one case the cell free homogenate was frozen at -15° C for one month prior to its use, and in the other a freshly prepared homogenate was heated at 50° C for 1 hour. Freezing completely destroyed the 17α-hydroxylase activity and reduced the 20-reductase activities by approximately 75%. Heating the homogenate completely destroyed the steroid metabolizing capabilities of the preparation within the limits of detection.

B. Comparison of buffers. The first experiments (Table III) were done with an incubation medium consisting of a 1:1 mixture of Krebs-Ringer-bicarbonate buffer and chicken or bovine serum (K-R-HCO_3:C.S. or K-R-HCO_3:B.S.), respectively. Both these media, especially that containing chicken serum, were cumbersome due to the large amount of fat extracted with the organic solvents after the incubation. The extracted fat severely hindered chromatographic separations. An experiment was therefore performed to compare the metabolism in the above two media and in plain Krebs-Ringer-phosphate buffer (K-R-PO_4) and Krebs-Ringer-bicarbonate buffer (K-R-HCO_3) (Table VII). The K-R-PO_4 buffer was used in subsequent studies, since the degree of progesterone metabolism was greatest in this medium.

C. Cofactor requirement. The cofactors included in the initial incubations were ATP, DPN, and TPN in 0.4 mM concentrations.

Table VI. Comparison of progesterone metabolism by frozen, heated, and untreated testis tissue cell-free homogenates. Testis tissue from birds stimulated Mar. 18-May 5 with an 18 hour daily photoperiod.
Average testicular weight: 408 mg.
Substrate: 0.01 μmole progesterone-4-C^{14}; 10 mc/mmole.
Final incubation volume: 10 ml.

	Average Conversion (% of control) Metabolites		
Pretreatment	V + V-Ac	VI + VI-Ac	VII
None	100	100	100
Frozen	0*	28.6	24.5
Heated (50°C-1 hr)	0*	0*	0*

* below limits of detection.
V= 17α-hydroxyprogesterone
V-Ac= Testosterone
VI= Androstenedione
VI-Ac= 4-pregnen-20α-ol-3-one
VII= 4-pregnen-20β-ol-3-one

Table VII. Effects of the buffer and medium on progesterone metabolism by testis tissue homogenates.
Testes from birds stimulated Nov. 16-Dec. 10 with an 18 hour daily photoperiod.
Average testicular weight: 262.5 mg.
Substrate: 0.05 μmoles progesterone-4-C^{14}; 2.0 mc/mmole.
Final incubation volume: 10 ml.

Buffer	Average Conversion (%) Metabolites		
	V + V-Ac	VI + VI-Ac	VII
K-R-HCO_3:C.S.	2.8	5.6	29.8
K-R-HCO_3:B.S.	4.2	4.8	18.7
K-R-HCO_3	5.5	6.5	24.5
K-R-PO_4	9.2	9.6	18.4

K-R-HCO_3:C.S.= Krebs-Ringer bicarbonate:Chicken serum (1/1)
K-R-HCO_3:B.S.= Krebs-Ringer bicarbonate:Bovine serum (1/1)
K-R-HCO_3= Krebs-Ringer bicarbonate buffer
K-R-PO_4= Krebs-Ringer phosphate buffer
V= 17α-hydroxyprogesterone
V-Ac= Testosterone
VI= Androstenedione
VI-Ac= 4-pregnen-20α-ol-3-one
VII= 4-pregnen-20β-ol-3-one

Nicotinamide (40 mM) and fumarate (1.0 mM) were also added to the medium. The adequacy of these arbitrary concentrations, and the necessity of the presence of these particular cofactors was investigated. It is evident from Tables VIII and IX that the concentrations of the cofactors and cosubstrates were apparently sufficient for maximal progesterone metabolism; doubling this concentration had no effect on the results. TPN was the only true cofactor required for full activity (Table IX). The presence of fumarate was also necessary, presumably to stimulate TPN reduction (171, 172). Nicotinamide was included at the 40 mM concentration in all incubations to inhibit the hydrolysis of pyridine nucleotides.

D. Temperature effect. The use of a 41° C incubation temperature seemed completely justified on a physiological basis, since this is the measured avian testicular temperature (234). However, a comparison of results obtained at 41° C and 37° C showed the total 17α-hydroxylase (V plus V-Ac) was greater at 37° C and the total progesterone 20-reductase was decreased (VI-Ac plus VII) (Table X). In spite of this data the 41° C temperature was retained in light of the physiological temperature measurements.

E. Identification of metabolites.

1. Metabolites I and II (Figure 6). These two incubation products

Table VIII. Effect of cofactor concentration on progesterone metabolism by cell-free testis tissue homogenates. Testes from birds stimulated Jan. 10-Feb. 6 with an 18 hour daily photoperiod.
Average testicular weight: 355.6 mg.
Substrate: 0.5 μmoles progesterone-4-C^{14}; 0.2 mc/mmole.
Final incubation volume: 10 ml.

Relative Cofactor Concentration	Average Conversion (%) Metabolites		
	V + V-Ac	VI + VI-Ac	VII
0 **	0 ***	0.60	0 ***
1 *	2.1	10.3	13.4
2	2.0	8.7	11.8

* ATP, DPN, and TPN: 0.4 mM; sodium fumarate: 1.0 mM; Nicotinamide: 40 mM.
** Nicotinamide conc. 40 mM in all cases.
*** below level of detection.
V = 17α-hydroxyprogesterone.
V-Ac = Testosterone
VI = Androstenedione
VI-Ac = 4-pregnen-20α-ol-3-one
VII = 4-pregnen-20β-ol-3-one

Table IX. Cofactor requirement for progesterone metabolism and androgen biosynthesis by cell-free testis tissue homogenate.
Testes from birds stimulated with 18 hr daily photoperiods for 1-4 weeks in Jan. and Feb.
Average testicular weight: 201 mg.
Substrate: 0.4 μmoles progesterone-4-C^{14}; 0.25 mc/mmole.
Final incubation volume: 10 ml.

Cofactor Omitted	Average Conversion (% of Control) Metabolites			
	V	V-Ac	VI-Ac	VII
None	100	100	100	100
ATP	108	76.6	104	107
DPN*	110	75.0	75.5	122
TPN**	103	123	132	72.1
TPN	76.5	26.7	33.2	28.6
Fumarate	71.0	4.78	23.7	25.3

* average of two separate incubations.
** TPN replaced by TPNH.
V = 17α-hydroxyprogesterone.
V-Ac = Testosterone.
VI-Ac = 4-pregnen-20α-ol-3-one.
VII = 4-pregnen-20β-ol-3-one.

Table X. Effect of incubation temperature on progesterone metabolism by cell-free testis tissue homogenates. Testes from birds stimulated Jan. 1-21 with 18 hour daily photoperiods.
Average testicular weight: 176.6 mg.
Substrate: 0.01 μmoles progesterone-4-C^{14}; 10 mc/mmole.
Final incubation volume: 10 ml.

Incubation Temperature	Average Conversion (%) Metabolites				
	V	V-Ac	VI	VI-Ac	VII
41° C	2.0	19.6	0.17	0.30	4.6
37° C	2.4	35.5	0.37	0.66	2.8

V = 17α-hydroxyprogesterone
V-Ac = Testosterone
VI = Androstenedione
VI-Ac = 4-pregnen-20α-ol-3-one
VII = 4-pregnen-20β-ol-3-one

were: the only two major metabolites not completely identified; the two most polar metabolites isolated; and chemically very similar, as judged by their chromatographic behavior before and after oxidation.

Table XI depicts the available chromatographic evidence on the relative polarity of these two compounds. Both metabolites were formed at essentially the same rate (0.1 mμmoles/hr/100 mg tissue), from progesterone -21-C^{14} and progesterone-4-C^{14} and were not formed from 17α-hydroxyprogesterone, androstenedione or testosterone. Metabolite I, the more polar of the two, was formed at a faster rate (0.3 mμmoles/hr/100 mg tissue) from 4-pregnen-20β-ol-3-one-4-C^{14} than from progesterone in one experiment.

As shown in Table XI, acetylation with the 15:1 pyridine:acetic anhydride solution gave three products while only the least polar was produced by the 4:1 solution. Oxidation of metabolite I or II produced compounds very similar, but not identical, in polarity to 6-keto-progesterone.

These data are consistant with the suggestion that these two metabolites are 21 carbon steroids with two acetylable hydroxyl groups, one at position 20 and the second at a position in the steroid nucleus.

2. Metabolite III (Figure 6). The chromatographic evidence supporting the identity of this product is shown in Table XII. The running rates of the unaltered product were suggestive of 4-pregnen-

Table XI. Chromatographic mobilities of metabolites I and II.

Paper Chromatographic System	Prior Treatment	R_f or R_x Metabolite I	Metabolite II	Internal Carrier
Chloroform/form.	none	0.28	0.49	
Chloroform/form.	"	0.61*	--	4-pregnen-6β,20α-diol-3-one
Chloroform/form.	"	--	0.93*	4-pregnen-6β,20β-diol-3-one
Bush, B-5 (5 hr)	"	0.86*	--	4-pregnen-6β,20α-diol-3-one
Bush, B-5 (5 hr)	"	--	0.50*	4-pregnen-6β,20β-diol-3-one
Benzene/form.	"	0.06	0.17	
Benzene/form.	"	0.68*	2.1*	11β-hydroxyandrostene-dione
Benzene/form.	"	0.57*	--	4-pregnen-6β,20α-diol-3-one
Benzene/form.	"	--	0.78*	4-pregnen-6β,20β-diol-3-one
Benzene/form. (10 hr. overrun)	"	3.8*	--	cortisone
Benzene/form.	Ac(4/1)	0.82	--	
Benzene/form.	Ac(15/1)	0.05**	--	
		0.44***	--	
		0.62****	--	
		0.82*****	--	
Hexane:benzene(1/1) /form.	Ox	0.53	0.52	
Hexane:benzene(1/1) /form.	Ox	1.06*	1.04*	6-ketoprogesterone

* Rx=mobility relative to internal carrier.
** representing 0.12% of the acetylated metabolite I.
*** representing 3.8% of the acetylated metabolite I.
**** representing 12.0% of the acetylated metabolite I.
***** representing 82.8% of the acetylated metabolite I.

Table XII. Chromatographic identification of metabolite III.

Paper Chromatographic System	Prior Treatment	R_f or R_x		
		C^{14} Metabolite	4-pregnen-17α, 20α-diol-3-one	
			As Internal Carrier	On Parallel Strip
Hexane:benzene (1/1) /form.	none	1.00*	--	--
Benzene/form. (3 hr overrun)	"	1.00*	--	--
Benzene/form.	"	0.28	--	0.28
Hexane:benzene(1/1) /form.	Ac	0.62	--	--
Hexane:benzene(1/1) /form.	Ox	0.69**	0.69	--
		0.35***	0.35	--

* R_x=mobility relative to internal carrier.
** representing 74-80% of the oxidized metabolite III. Androstenedione carrier.
*** representing 20-26% of the oxidized metabolite III. 17α-hydroxyprogesterone carrier.
Ac= acetylated.
Ox= oxidized.
form.= formamide.

17α, 20α-diol-3-one. Acetylation produced a compound of lower polarity consonant with the formation of a monoacetate derivative. Oxidation of the "free" metabolite yielded a major product (75%) with a chromatographic mobility identical to androstenedione and a minor product (25%) of chromatographic mobility identical to 17α-hydroxyprogesterone.

Retention times relative to cholestane on a packed 6 foot gas chromatographic column were identical (1.57) for the biosynthetic compound III and authentic 4-pregnen-17α, 20α-diol-3-one.[1] Elution patterns from micro-silica gel columns were consistent for biosynthetic and authentic material (Figure 11). Although sufficient material of the 20α isomer was not available to obtain a sulfuric acid spectrum, the spectrum of metabolite III was very similar to authentic 4-pregnen-17α, 20β-diol-3-one. The major maxima occurred at 291 mμ and 299 mμ for metabolite III and the synthetic steroid respectively; the major minima were at 245-246 and 236 mμ. It is not highly likely that the sulfuric acid spectra would distinguish 20α and 20β isomers (242).

Metabolite III was formed from 4 or 21-C^{14}-progesterone and at a faster rate from 17α-hydroxyprogesterone. It was, of course, not formed when the incubation substrate was either testosterone or androstenedione-4-C^{14}. It also absorbed ultraviolet light in the 240mμ region.

[1]Obtained from Dr. D. R. Idler, Fisheries Research Board, Vancouver, B. C.

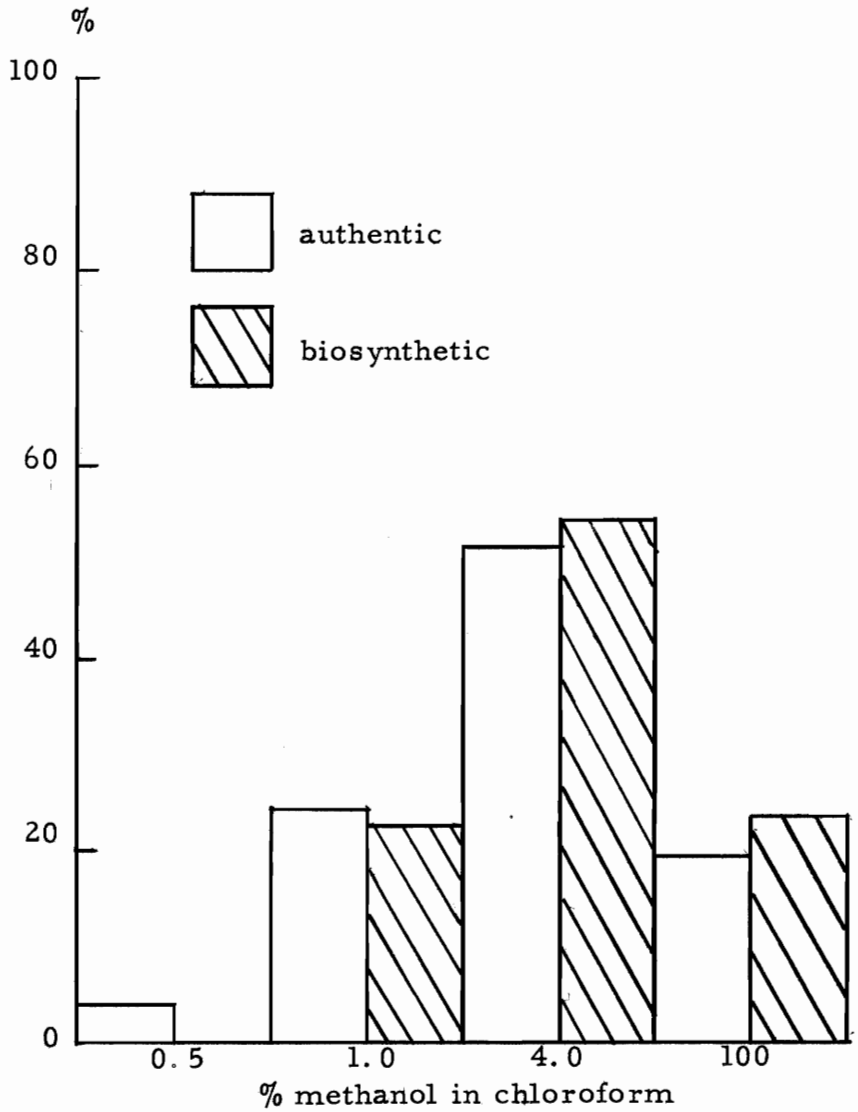


Figure 11. Elution patterns of authentic and biosynthetic 4-pregnen-17α, 20α-diol-3-one from micro-silica gel columns.

By these various criteria the identity of metabolite III was quite definitely established as 4-pregnen-17α, 20α-diol-3-one.

3. Metabolite IV (Figure 6). Table XIII shows the chromatographic evidence for the identity of metabolite IV with 4-pregnen-17α, 20β-diol-3-one. This metabolite behaved similarly to metabolite III and it also appeared to form a monoacetate derivative on acetylation. However, the free compound was slightly less polar than metabolite III, and its only oxidation product had a mobility identical to authentic androstenedione as compared to the dual product formation on oxidation of metabolite III.

The retention times relative to cholestane on the gas column were identical (1.46) for this metabolite and authentic 4-pregnen-17α, 20β-diol-3-one, and the sulfuric acid spectrum was indistinguishable from metabolite III.

A micro infra-red analysis showed the spectra of metabolite IV and 4-pregnen-17α, 20β-diol-3-one to be almost superimposable (Figure 12).

This metabolite was formed from the same substrates as metabolites III and also had a UV absorption maximum around 240 mμ.

By these various parameters, the most unequivocal of which is probably the infrared analysis, the identity of metabolite IV was established as 4-pregnen-17α, 20β-diol-3-one.

4. Metabolite V (Figure 6). Complete paper chromatographic evidence for the identity of metabolite V as 17α-hydroxyprogesterone is

Table XIII. Chromatographic identification of metabolite IV.

Paper Chromatographic System	Prior Treatment	R_f		
		C^{14} Metabolite	4-pregnen-17α, 20β-diol-3-one As Internal Carrier	4-pregnen-17α, 20β-diol-3-one On Parallel Strip
Benzene/form.	none	0.34	--	0.35
Bush, B-3	"	0.31	--	0.30
Hexane:benzene(1/1) /form.	Ac	0.68	0.67	--
Hexane:benzene(1/1) /form.	Ox	0.68*	0.69	--

* representing 100% of the oxidized metabolite IV.
Androstenedione carrier.
Ac= acetylated.
Ox= oxidized.
form.= formamide.

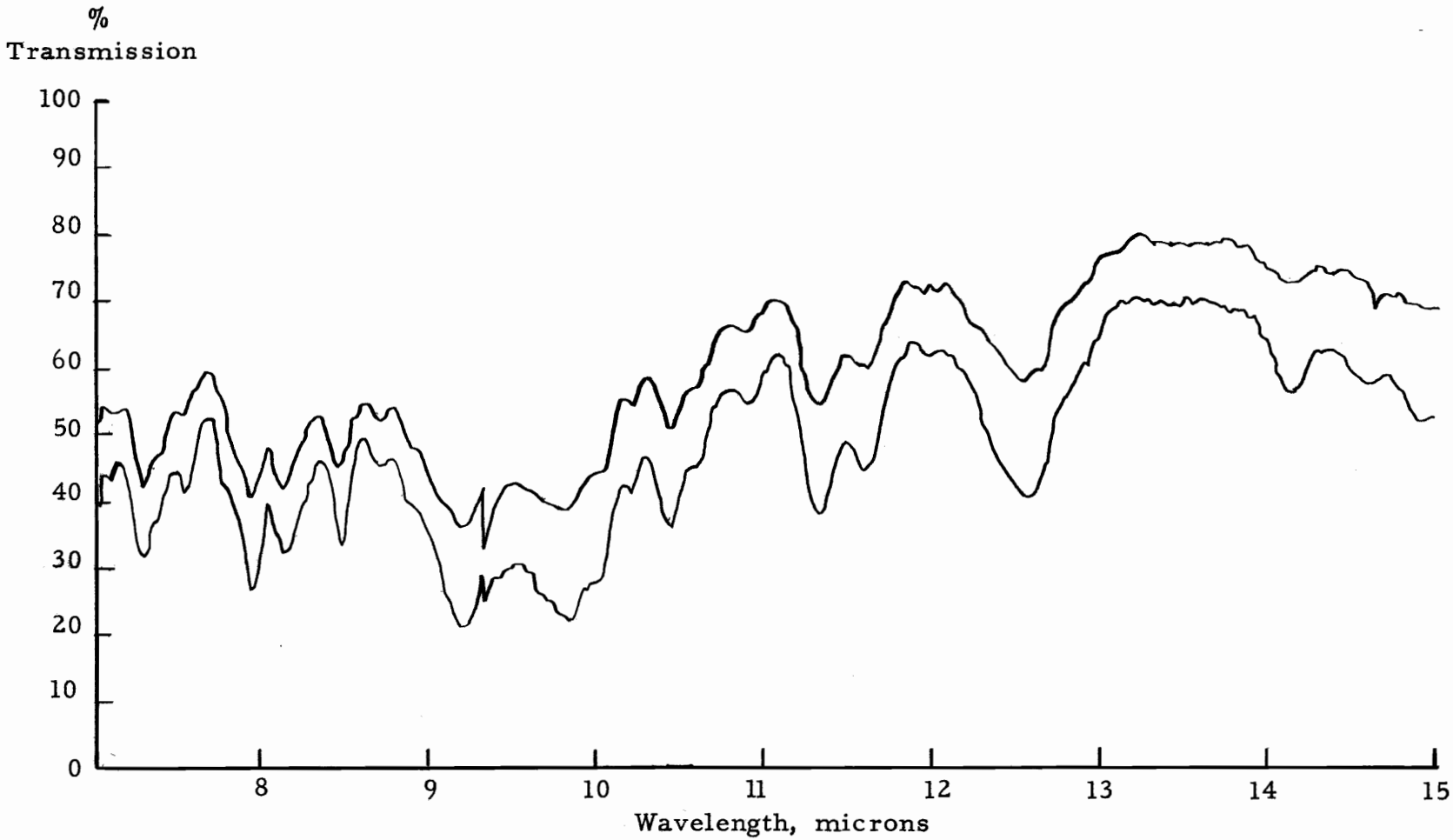


Figure 12. Infra-red absorption spectra of authentic 4-pregnen-17α, 20β-diol-3-one (lower curve) and biosynthetic metabolite (upper curve).

presented in Table XIV. Acetylation of this metabolite together with authentic 17α-hydroxyprogesterone did not alter the mobility of either in three different paper chromatography systems. The major product (approx. 90%) after oxidation also showed no change in chromatographic mobility; approximately 10% had a mobility identical to androstenedione.

The first two columns in Table XV show the data obtained after recrystallization of the C^{14} labeled metabolite V with added authentic 17α-hydroxyprogesterone. As can be seen, within the limitations of this technique, the specific activity becomes constant with repeated crystallization of the steroid.

The conclusion that 17α-hydroxyprogesterone and metabolite V are chemically identical seems irrefutable.

5. Metabolite V-Ac (Figure 6). A thorough identification by paper chromatography of metabolite V-Ac and its oxidation and acetylation products established this compound as testosterone. These results are shown in Table XVI. When authentic testosterone was added to the radioactive metabolite, the mobilities of the two were indistinguishable, whether prior to treatment, following acetylation, or after oxidation of the saponified acetate derivative.

Authentic testosterone acetate was added to the acetate derivative of this metabolite and the mixture recrystallized to constant specific activity (Table XV). This confirmed the chromatographic identification.

Table XIV. Chromatographic identification of metabolite V.

Paper Chromatographic System	Prior Treatment	R_f or R_x		
		C^{14} Metabolite	17α-hydroxyprogesterone As Internal Carrier	17α-hydroxyprogesterone On Parallel Strip
Hexane:benzene (1/1) /form.	none	0.31	0.31	--
Benzene/form.	"	0.58	0.57	0.59
Hexane:benzene(1/1) /form.	Ac	0.26	0.26	--
Benzene/form.	"	0.59	0.58	--
Bush, B-3 (13.5 hr)	"	1.00*	--	--
Hexane:benzene(1/1) /form.	Ox**	0.30	0.30	--
Benzene/form.	"	0.52	0.51	--
Bush, B-3 (13 hr)	"	0.99*	--	--

*= R_x= mobility relative to internal carrier.
Ac= acetylated.
Ox= oxidized.
Form.= formamide.
** mobilities given are for the major oxidation product; approx. 10% of the C^{14} metabolite oxidized had a mobility similar to androstenedione in all three systems.

Table XV. Recrystallization of C^{14}-metabolites to constant specific activity.

Recrystallization	17α-OH-progesterone		Androstenedione		Testosterone Acetate		4-pregnen-20β-ol-3-one	
	Solvent	Specific Activity cpm/mg.	Solvent	Specific Activity cpm/mg.	Solvent	Specific Activity cpm/mg.	Solvent	Specific Activity cpm/mg.
0	---	1545	---	---	---	7645	---	1069
1	EtOH/ Pet.Et.	1522	MeOH/ Et_2O	564	MeOH/ hexane	8432	MeOH/ H_2O	1097
2	EtOH/ Acetone	1613	EtOH	634	MeOH/ hexane	9160	EtOAc/ hexane	1212
3	MeOH/ Acetone	1492	EtOH/ H_2O	583	MeOH/ H_2O	9080	MeOH/ H_2O	1123
4	MeOH/ Et_2O	1579	---	---	---	---	---	---

Table XVI. Chromatographic identification of metabolite V-Ac.

Paper Chromatographic System	Prior Treatment	R_f or R_x		
		C^{14} Metabolite	Testosterone As Internal Carrier	Testosterone On Parallel Strip
Hexane:benzene (1/1) /form.	none	0.31	0.31	--
Benzene/form.	"	0.58	0.57	0.59
Hexane/form. (24-40 hr)	"	1.00*	--	--
Bush, B-3 (13 hr)	Ac & Sap	1.01*	--	--
Hexane:benzene (1/1) /form.	" " "	0.30	0.30	--
Hexane:benzene(1/1) /form.	Ac	0.91	0.87	0.84
Hexane/form.	"	0.65	0.65	0.65
Methylcyclohexane/ P.G.	"	0.61	0.61	--
Bush-A (13.5 hr)	"	0.99*	--	--
Hexane:benzene(1/1) /form.	Ac,Sap, & Ox	0.64	0.64	0.66
Hexane/form.	" "	0.12	0.12	--
Methylcyclohexane/ P.G.	" "	0.13	0.12	--

* R_x = mobility relative to internal carrier.
Ac = acetylated.
Sap = saponified.
Ox = oxidized.
Form.= formamide.
P.G. = propyleneglycol.

This compound could be isolated after incubations with progesterone-4-C^{14}, 17α-hydroxyprogesterone-4-C^{14}, androstenedione-4-C^{14}, or testosterone-4-C^{14} substrates, but not in detectable amounts using progesterone-21-C^{14} [redacted] as incubation substrates.

6. Metabolite VI (Figure 6). This compound ran with the androstenedione carrier in the first chromatographic separation of incubation metabolites. It was, however, usually a minor component of the radioactivity in this area of the chromatogram. After acetylation of the eluted androstenedione area, two C^{14}-metabolites were separated. The major component formed an acetate derivative, while the minor component did not and, as shown in Table XVII, continued to move with the androstenedione carrier in several chromatographic systems. These mobilities were not changed after chromic acid oxidation.

The second column in Table XV shows that the specific activity was relatively constant through three crystallizations of this metabolite with authentic androstenedione.

As might be expected this compound was formed from progesterone-4-C^{14}, 17α-hydroxyprogesterone-4-C^{14}, and testosterone-4-C^{14} substates, but not in detectable amounts from 4-pregnen-20β-ol-3-one-4-C^{14} or progesterone-21-C^{14}..

On the strength of the chromatographic evidence, the effects of

Table XVII. Chromatographic identification of metabolite VI.

Paper Chromatographic System	Prior Treatment	R_f or R_x		
		C^{14} Metabolite	Androstenedione	
			As Internal Carrier	On Parallel Strip
Hexane/form.	Ac	0.10	0.10	--
Hexane:benzene(1/1) /form.	"	0.61	0.61	--
Bush, B-3 (13.5 hr)	"	0.99*	--	--
Hexane/form.	Ox	0.097	0.097	--
Hexane:benzene (1/1) /form.	"	0.63	0.62	--
Bush, B-3 (13 hr)	"	1.00*	--	--

* R_x = mobility relative to internal carrier.
Ac = acetylated.
Ox = oxidized.
Form. = formamide.

oxidation and acetylation, the evidence for the presence of 19 rather than 21 carbons, and strongly supported by the crystallization data, metabolite VI was identified as androstenedione.

7. Metabolite VI-Ac (Figure 6). Metabolite VI-Ac is defined as that part of the C^{14} accompanying the authentic androstenedione carrier in the original chromatogram which separated from the androstenedione by formation of an acetate derivative when treated with acetic anhydride in pyridine. Chromatographic mobilities of the parent compound and its derivatives together with similarly treated 4-pregnen-20α-ol-3-one carrier are shown in Table XVIII. The mobilities of metabolite and 4-pregnen-20α-ol-3-one are indistinguishable. It should be noted that the product of oxidation of the saponified acetate derivative has mobilities very similar to those of progesterone (Table XX).

Attempts to recrystallize this material to constant specific activity were uniformly unsuccessful. The specific activity decreased slightly after each crystallization. Overall decreases in specific activity from the starting material to the final crystallization product of 20%, 26%, and 32% for 3, 4, and 4 recrystallizations, respectively, were observed.

This metabolite was formed only when progesterone-4-C^{14} or 21-C^{14} were the substrates in the incubations and could be seen to have

Table XVIII. Chromatographic identification of metabolite VI-Ac.

Paper Chromatographic System	Prior Treatment	R_f or R_x		
		C^{14} Metabolite	4-pregnen-20α-ol-3-one As Internal Carrier	4-pregnen-20α-ol-3-one On Parallel Strip
Hexane:benzene(1/1) /form.	none	0.72	0.72	--
Hexane/form. (8 hr overrun)	"	1.00*	--	--
Methylcyclohexane/ P.G.	Ac & Sap	0.058	0.060	--
Hexane/form.	"	0.11	0.12	--
Hexane/form.	Ac	0.74	0.73	0.74
Hexane/P.G.	"	0.55	0.55	--
Bush-A	"	0.45	0.44	--
Methylcyclohexane/ P.G.	"	0.51	0.55	--
Hexane:benzene(1/1) /form.	Ac, Sap, & Ox	0.78	0.78	--
Hexane/form.	" "	0.41	0.41	--
Methylcyclohexane/ P.G.	" "	0.32	0.32	--

* R_x = mobility relative to internal carrier.
Ac = acetylated.
Sap = saponified.
Ox = oxidized.
Form. = formamide.
P.G. = propyleneglycol.

U. V. absorption when large substrate concentrations were used and no carriers added to the first chromatogram.

Notwithstanding the recrystallization data, the chromatographic evidence for the formation of metabolite V by the reduction of the 20 ketone of progesterone to the 20α-hydroxyl group seems quite good. A possible explanation for the inability to crystallize this material to constant specific activity could be that this metabolite was difficult to obtain in a pure form. If the metabolite was purified chromatographically as isolated from the incubations, it could not be obtained free from contaminating metabolite VI, androstenedione. If it were separated from the androstenedione by acetylation there was always a fair chance that it was contaminated with the 20β isomer, which was always present in larger amounts than the 20α. These two isomers were separable chromatographically but whether or not the separation was quantitative is open to question. Also there is the possibility that the synthetic 4-pregnen-20α-ol-3-one, which admittedly contained a minor contaminant of the 20β isomer (~5%), could have been impure enough to cause these spurious results.

8. Metabolite VII (Figure 6). Metabolite VII was the seventh radioactive peak in order of decreasing polarity on the original chromatogram. It was located between the added androstenedione carrier and the non-metabolized progesterone substrate and was formed only from this

substrate, both 4 and 21-C^{14} labeled.

The chromatographic mobilities are presented in Table XIX. The metabolite appeared identical to authentic 4-pregnen-20β-ol-3-one by these criteria. In addition the mobilities of the oxidation product were very similar to those of progesterone (Table XX).

Recrystallization to constant specific activity was accomplished after the addition of authentic 4-pregnen-20β-ol-3-one to the metabolite as shown in column four of Table XV. These data confirmed the chromatograph identification of this metabolite as 4-pregnen-20β-ol-3-one.

9. Metabolite VIII (Figure 6). This radioactive peak was the least polar major "metabolite." It was present in the same area of the chromatogram as the progesterone substrate from blank incubations and zero time controls and was found only when the substrate was progesterone-4 or 21-C^{14} or 4-pregnen-20β-ol-3-one-4-C^{14}. Confirmation of its identity with progesterone is shown in Table XX. The chromatographic mobilities were unchanged after acetylation and oxidation as were those of the authentic progesterone carrier.

Briefly recounting, nine metabolites have been isolated and tentatively or completely identified from incubations with sparrow testicular tissue. In order of decreasing polarity these are: two incompletely identified, relatively polar compounds which appear to be dihydroxy compounds with one hydroxyl group at the 20 position and a

Table XIX. Chromatographic identification of metabolite VII.

Paper Chromatographic System	Prior Treatment	R_f or R_x		
		C^{14} Metabolite	4-pregnen-20β-ol-3-one As Internal Carrier	4-pregnen-20β-ol-3-one On Parallel Strip
Hexane:benzene(1/1) /form.	none	0.66	0.65	0.65
Hexane/form.	"	0.21	0.21	0.20
Heptane/form.	"	0.20	0.20	0.19
Methylcyclohexane/ P.G.	"	0.11	0.12	--
Hexane/form.	Ac	0.80	0.79	0.80
Methylcyclohexane/ P.G.	"	0.73	0.71	--
Bush-A (13 hr)	"	0.99*	--	--
Hexane/form.	Ox	0.45	0.45	--
Methylcyclohexane/ P.G.	"	0.57	0.57	--
Bush-A (13 hr)	"	1.01*	--	--

* = R_x = mobility relative to internal carrier.
Ac = acetylated.
Ox = oxidized.
Form. = formamide.
P.G. = propyleneglycol.

Table XX. Chromatographic identification of metabolite VIII.

Paper Chromatographic System	Prior Treatment	R_f or R_x		
		C^{14} Metabolite	Progesterone As Internal Carrier	Progesterone On Parallel Strip
Hexane:benzene(1/1) /form.	none	0.80	0.80	--
Methylcyclohexane, /P.G.	"	0.41	0.41	--
Bush-A (13.5 hr)	"	1.00*	--	--
Methylcyclohexane, /P.G.	Ac	0.43	0.43	--
Bush-A (13 hr)	"	0.99*	--	--
Hexane:benzene(1/1) /form.	Ox	0.79	0.78	--
Hexane/form.	"	0.48	0.47	--
Methylcyclohexane /P.G.	"	0.43	0.43	--
Bush-A (13 hr)	"	1.00*	--	--

* = R_x = mobility relative to internal carrier.
Ac = acetylated.
Ox = oxidized.
Form. = formamide.
P.G. = propyleneglycol.

second at some acetylable location in the steroid nucleus; 4-pregnen-17α, 20α-diol-3-one; 4-pregnen-17α, 20β-diol-3-one; 17α-hydroxyprogesterone; testosterone; androstenedione; 4-pregnen-20α-ol-3-one, which has not been successfully crystallized to constant specific activity with authentic material; and 4-pregnen-20β-ol-3-one.

The average per cent conversion of 0.01 μmole of progesterone-4-C^{14} to these metabolites is shown at the base of the radioactive chromatogram recording in Figure 6. The ratio of testosterone to 17α-hydroxyprogesterone (peak V) was quite variable. Since the tissue used in these qualitative incubations was obtained from birds stimulated to gonadal activity in various ways, the inconsistancies are, perhaps, understandable. The ratio of androstenedione to 4-pregnen-20α-ol-3-one (peak VI) was also variable. Generally the androstenedione accounted for less than 10% of the C^{14} in this area of the chromatogram.

F. Elucidation of metabolic pathways.

1. Incubations with progesterone as substrate. All the products discussed in the previous section were isolated with progesterone substrate. Results obtained with other C^{14}-labeled substrates were compared to those from the progesterone substrate incubations. A rather obvious assumption was made that three of the isolated compounds, 4-pregnen-20α-ol-3-one, 4-pregnen-20β-ol-3-one and 17α-hydroxyprogesterone were direct products of progesterone metabolism without the formation

of "free" intermediates.

2. Incubations with 4-pregnen-20β-ol-3-one as substrate. In such incubations the rate of production of metabolite I was greater than with the same concentration (0.01 μmole /10 ml) of progesterone as substrate. Three-tenths of a millimicromole of metabolite I were formed from progesterone in a three hour period and 0.98 millimicromoles from 4-pregnen-20β-ol-3-one. In addition, progesterone, 17α-hydroxyprogesterone, and testosterone were formed in small but significant amounts. This tends to demonstrate that only one product, metabolite I, was formed from progesterone via 4-pregnen-20β-ol-3-one and that the 20β reduction of progesterone is reversible under these conditions.

3. Incubations with 17α-hydroxyprogesterone as substrate. Use of 4-C^{14}-17α-hydroxyprogesterone as substrate led to the formation of 4-pregnen-17α, 20α-diol-3-one, 4-pregnen-17α, 20β-diol-3-one, and testosterone at a faster rate than with progesterone as substrate at the same concentration (Table XXI). Androstenedione formation was not quantitated in this incubation. Little, if any, metabolite I or II, progesterone, and the 20 reduced progesterones were formed from 17α-hydroxyprogesterone.

This evidence suggested that the 20 reduction occurred following 17 hydroxylation in metabolites III and IV and that 17 hydroxylation preceeded the removal of carbons 20 and 21.

4. Incubations with androstenedione-4-C^{14} as substrate. Only one

Table XXI. Comparison of product formation from progesterone and 17α-hydroxyprogesterone by cell-free testis tissue homogenate.
Testes from normal birds trapped July 1-7.
Average testicular weight: 250 mg.
Substrates: 0.01 μmole progesterone-4-C^{14}; 10mc/mmole, or 0.01 μmole 17α-hydroxyprogesterone-4-C^{14}; 10 mc/mmole.
Final incubation volume: 10 ml.

Metabolite	mμmoles/3 hr. Substrate	
	Progesterone	17α-hydroxyprogesterone
I	0.42	0*
II	1.3	0*
III	0.60	2.5
IV	0*	1.6
V	0.10	0.33
V-Ac	0.52	1.7
VI	---	---
VI-Ac	1.0	0*
VII	2.1	0*
VIII	3.0	0*

I and II = incompletely identified products
III = 4-pregnen-17α,20α-diol-3-one
IV = 4-pregnen-17α,20β-diol-3-one
V = 17α-hydroxyprogesterone
V-Ac = Testosterone
VI = Androstenedione (not measured)
VI-Ac = 4-pregnen-20α-ol-3-one
VII = 4-pregnen-20β-ol-3-one
VIII = Progesterone
* = nondetectable

product, testosterone-4-C^{14}, was isolated in approximately 80% yield from 0.05 μmoles of androstenedione-4-C^{14}. It was interesting to note that with 0.01 μmole of progesterone, or 0.05 μmoles of androstenedione as substrate the final ratios of testosterone to androstenedione were 40 and 38 respectively. These results might have been expected if the metabolic pathway is similar to that in mammalian testicular tissue (212).

5. Incubation with testosterone as substrate. The only product formed from a testosterone-4-C^{14} substrate incubation was 4-C^{14}-androstenedione. The testosterone to androstenedione ratio in this incubation was 23 with the testosterone substrate (0.027 μmoles) and 33 with progesterone substrate (0.01 μmoles).

6. Incubations with unlabeled "traps" included. The results obtained with 1 micromole traps of 17α-hydroxyprogesterone, 4-pregnen-17α, 20α-diol-3-one, androstenedione, and testosterone with either progesterone-4-C^{14} or 17α-hydroxyprogesterone-4-C^{14} are presented in Tables XXII and XXIII, where the per cent of the metabolite formed is compared to that formed in incubation without such traps. It can be seen that with progesterone-4-C^{14} as substrate the presence of an unlabeled 17α-hydroxyprogesterone trap markedly decreased the C^{14} in metabolites formed from 17α-hydroxyprogesterone. Thus, the labeled 4-pregnen-17α, 20α-diol-3-one, 4-pregnen-17α, 20β-diol-3-one, testosterone, and androstenedione decreased while the C^{14}-17α-hydroxyprogesterone

Table XXII. Elucidation of pathways of steroid metabolism in testis tissue cell-free homogenates.
Testes from birds stimulated with:
* 18 hr photoperiod from Mar. 18-May 5.
** 18 hr photoperiod from Jan. 1-21.
*** 6750 IU HCG from Aug. 29-Sept. 13.
Average testicular weights: * 408 mg; ** 176.6 mg; and *** 55 mg.
Substrate: 0.01 μmole; 10 mc/mmole.
Trap: 1.0 μmole- unlabeled.
Final incubation volume: 10 ml.

		Average Conversion (% of control) Metabolite		
Substrate	Trap	17α-hydroxy-progesterone	Testosterone	Androstenedione
Progesterone	none	100	100	100
" *	17α-OH-progesterone	308	6.7	60
" *	Androstenedione	1967	71.5	400
" **	4-pregnen-17α,20α-diol-3-one	206	31.5	62.8
17α-OH-progesterone	none	100	100	100
" ***	Testosterone	78.6	115	217
" ***	Androstenedione	279	50.8	1909

Table XXIII. Elucidation of pathways of steroid metabolism in testis tissue cell-free homogenates.
Testes from birds stimulated with an 18 hr daily photoperiod from:
* Mar. 18 to May 5, or
** Jan. 1 to Jan 21.
Average testicular weights:
* 408 mg, or
** 176.6 mg.
Substrate: 0.01 μmole; 10 mc/mmole.
Trap: 1.0 μmole.
Final incubation volume: 10 ml.

Substrate	Unlabeled trap	Average Conversion (% of control) Metabolite I	II	4-pregnen-17α, 20α-diol-3-one	4-pregnen-17α, 20β-diol-3-one	4-pregnen-20α-ol-3-one	4-pregnen-20β-ol-3-one
Progesterone	none	100	100	100	100	100	100
" *	17α-OH-progesterone	15.1	14.1	18.5	16.4	24.5	970
" *	Androstenedione	17.9	26.6	76.5	36.4	65.5	150
" **	4-pregnen-17α,20α-diol-3-one	72.5	43.7	105	116	519	1495

increased. Effects on the formation of other metabolites were also noted. While metabolites I, II, and 4-pregnen-20α-ol-3-one are not formed via 17α-hydroxyprogesterone, the presence of 1.0 micromole of this steroid decreased the C^{14} entering these products from the progesterone substrate. However, the label in 4-pregnen-20β-ol-3-one increased.

An unlabeled androstenedione trap was used with progesterone-4-C^{14} substrate in an attempt to demonstrate whether androstenedione or testosterone is the immediate product formed from 17α-hydroxyprogesterone by this tissue. Table XXII shows that the C^{14} in 17α-hydroxyprogesterone and androstenedione increased, while in testosterone it decreased slightly. By measuring the U.V. absorbance of the isolated testosterone and androstenedione the amount of the unlabeled androstenedione trap which had been metabolized to testosterone during the 3 hour incubation period was determined. This was possible because the absolute amount of androstenedione and testosterone formed from the 0.01 μmole of progesterone substrate was negligible compared to that from the "cold" trap. It was found that better than 3/4 (0.77 μmoles) of the unlabeled androstenedione had been converted to testosterone. This raised the question whether the trap had actually been functioning as androstenedione or testosterone during the incubation. Other effects of this trap on the progesterone metabolism to products not formed via androstenedione were: decreases in C^{14}-metabolites I and II, 4-pregnen-

17α, 20αdiol-3-one, 4-pregnen-17α, 20β-diol-3-one, and 4-pregnen-20α-ol-3-one and an increase in the 4-pregnen-20β-ol-3-one-4-C^{14} formed.

Although it appeared unlikely from the previous results that either isomer of 20 reduced 17α-hydroxyprogesterone could be an intermediate between the androgens and 17α-hydroxyprogesterone, 17α, 20α-dihydroxyprogesterone trap was used in one experiment. The results (Tables XXII and XXIII) showed decreases in the C^{14}-androstenedione and testosterone and an increase in the labeled 17α-hydroxyprogesterone. However, there was no detectable U. V. absorbing material from the 17α, 20α-dihydroxyprogesterone trap in any of these metabolites. Thus, the C^{14} labeled androgens must have been formed directly from 17α-hydroxyprogesterone, and it was highly unlikely that they had come through the trap as an intermediate. The decreases in the amount of labeled testosterone and androstenedione formed was probably due to inhibition of the enzyme that cleaves the two carbon side chain from 17α-hydroxyprogesterone by the high concentration of 4-pregnen-17α, 20α-diol-3-one present as trap.

There was little effect on the amount of labeled 4-pregnen-17α, 20α-diol-3-one and 4-pregnen-17α, 20β-diol-3-one formed. A decrease in the amount of the C^{14} in metabolites I and II, and an increase in the C^{14} in the two isomers of 20 reduced progesterone were, however,

observed.

To clarify the sequence of reactions involving formation of androstenedione and testosterone from 17α-hydroxyprogesterone, incubation were performed using 0.01 μmole of 17α-hydroxyprogesterone-4-C^{14} substrate and 1.0 μmole traps of testosterone and androstenedione. The results (Table XXII) show that the testosterone trap caused an increase in the C^{14} in the isolated androstenedione and little change in testosterone. Virtually none of the testosterone trap was metabolized to androstenedione as determined by U.V. absorbance.

The androstenedione trap caused a much greater increase in the labeled androstenedione and a marked decrease in the testosterone formed from the 17α-hydroxyprogesterone-4-C^{14}. Three-fifths of the androstenedione trap material had been converted to testosterone by the end of the incubation. It should also be noted that the androstenedione trap caused a definite increase in the 17α-hydroxyprogesterone-4-C^{14} remaining at the end of the incubation period, whereas the testosterone trap had little effect in this respect. The results of these two trap experiments can be explained only if androstenedione precedes testosterone in the biosynthetic pathway and is the immediate product formed by removing the two carbon side chain unit from 17α-hydroxyprogesterone.

The trap experiments lend further support to the metabolic pathways shown in Figure 13. In every case that progesterone metabolism was

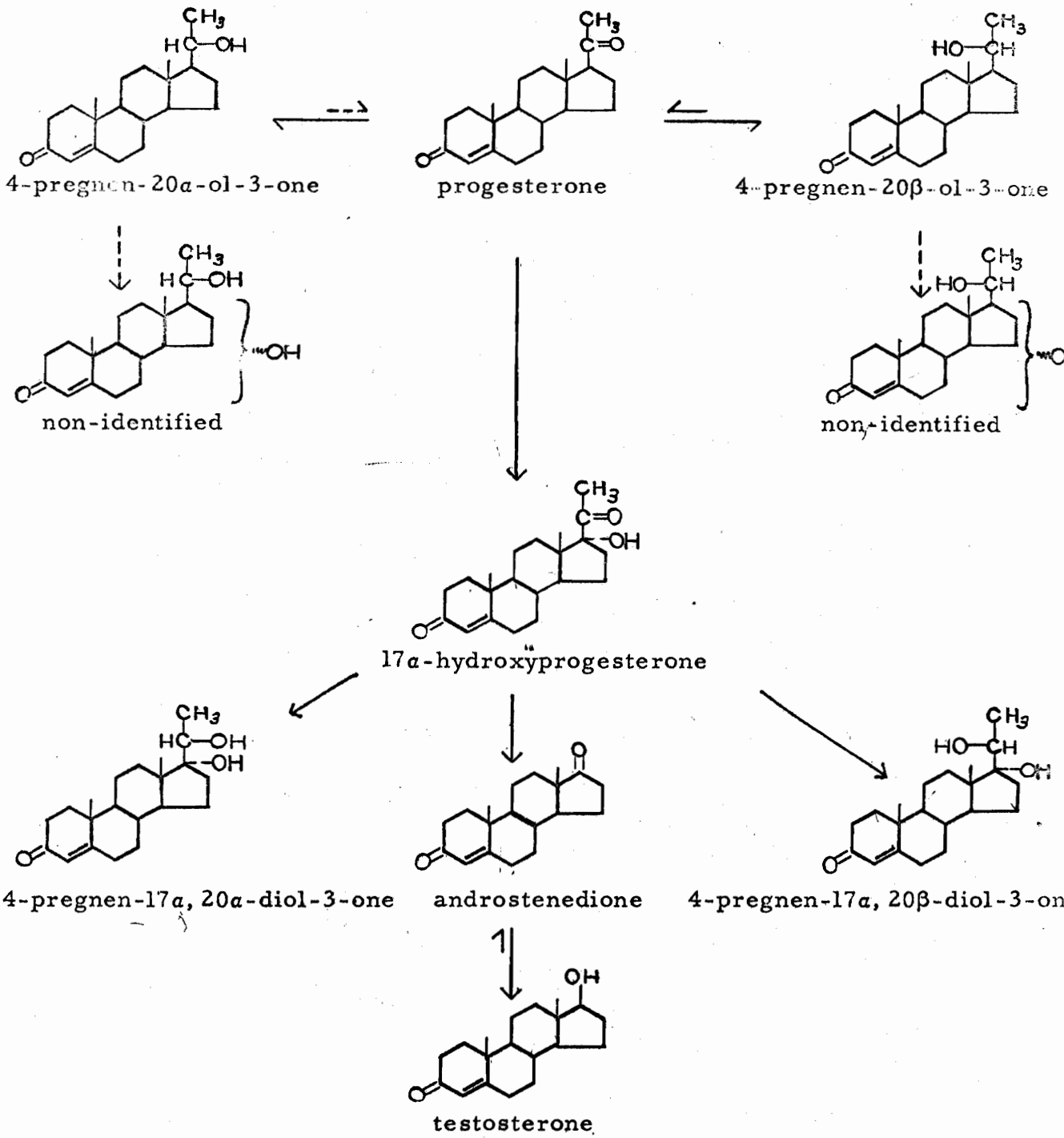


Figure 13. Proposed pathways of androgen biosynthesis and progesterone metabolism in English sparrow (Passer domesticus) testicular tissue.

blocked by a large amount of unlabeled steroid, the degree of 20β reduction of progesterone was increased-- presumably because more progesterone was available for this enzyme. Although the 20α reduction of progesterone increased in the presence of the 4-pregnen-17α, 20α-diol-3-one trap, it decreased when either androstenedione or testosterone traps were used. The only apparent explanation is an inhibition of the 20α-ketone reductase by these large amounts of steroids added as trap. This type of inhibition is also a possible explanation for the uniform decreases as seen in metabolites I and II with the addition of unlabeled steroids that were known not to be precursors of these metabolites.

G. Substrate concentration studies. Figure 14 shows the results of incubations performed at various substrate concentrations. It appears that the 17α-hydroxylase system is saturated first and the 20 reductases "remove" the excess progesterone and get only that which is left over. This substantiates evidence for this point presented earlier (Figure 8-10).

H. Time course study. An attempt was made to follow the time course of the progesterone metabolism as another approach to the study of the reaction sequence. Reactions were stopped at [redacted] 30, 60, 180, and 360 minutes, and the results shown in Figures 15 and 16. These curves were consistent with the metabolic pathway in Figure 13.

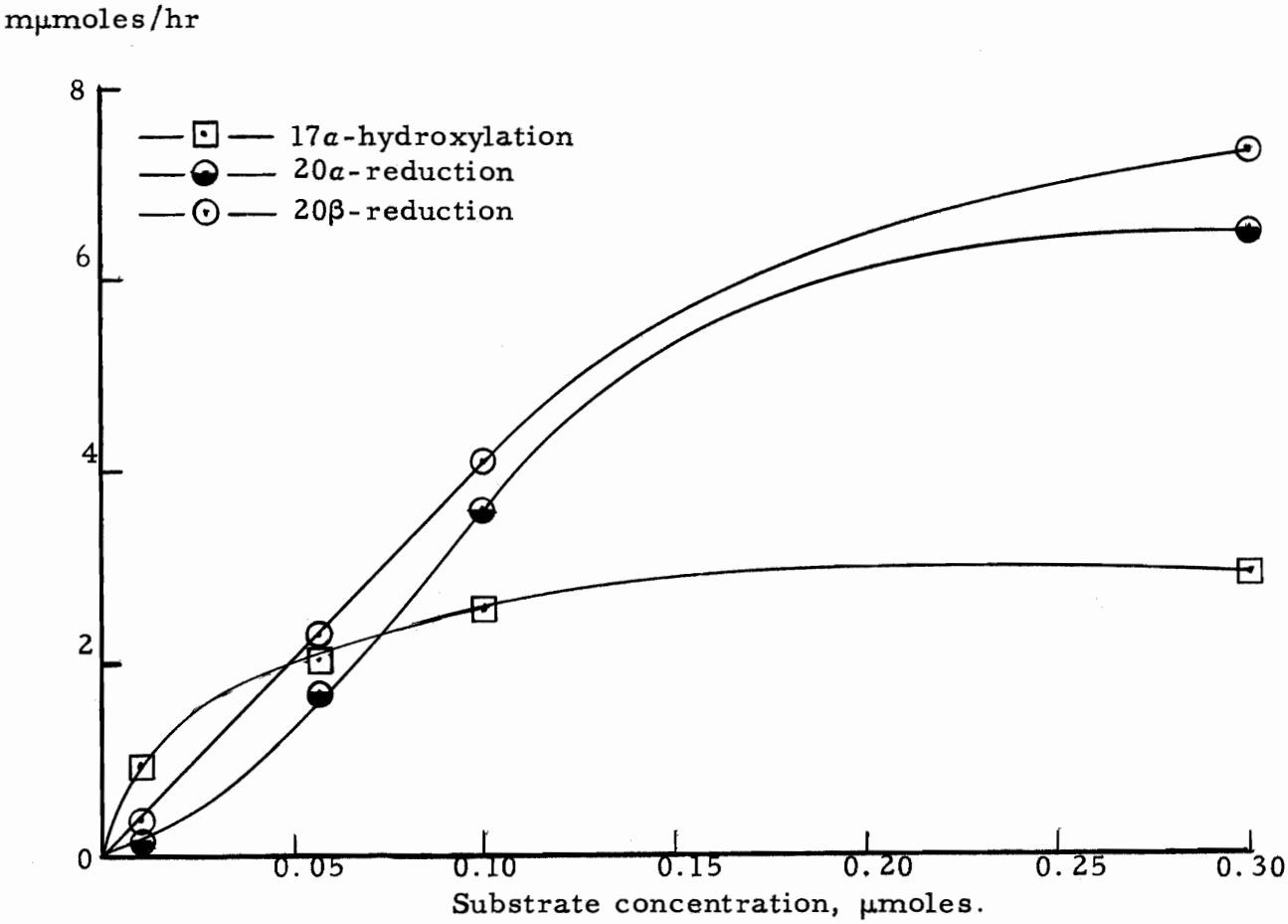


Figure 14. Variation of the rates of product formation with substrate concentration.
Testes from birds stimulated with an 18 hr. photoperiod from Feb. 6-Mar. 7.
Mean testicular weight: 291 mg.
Substrate: progesterone-4-C^{14},
Final incubation volume: 10 ml.

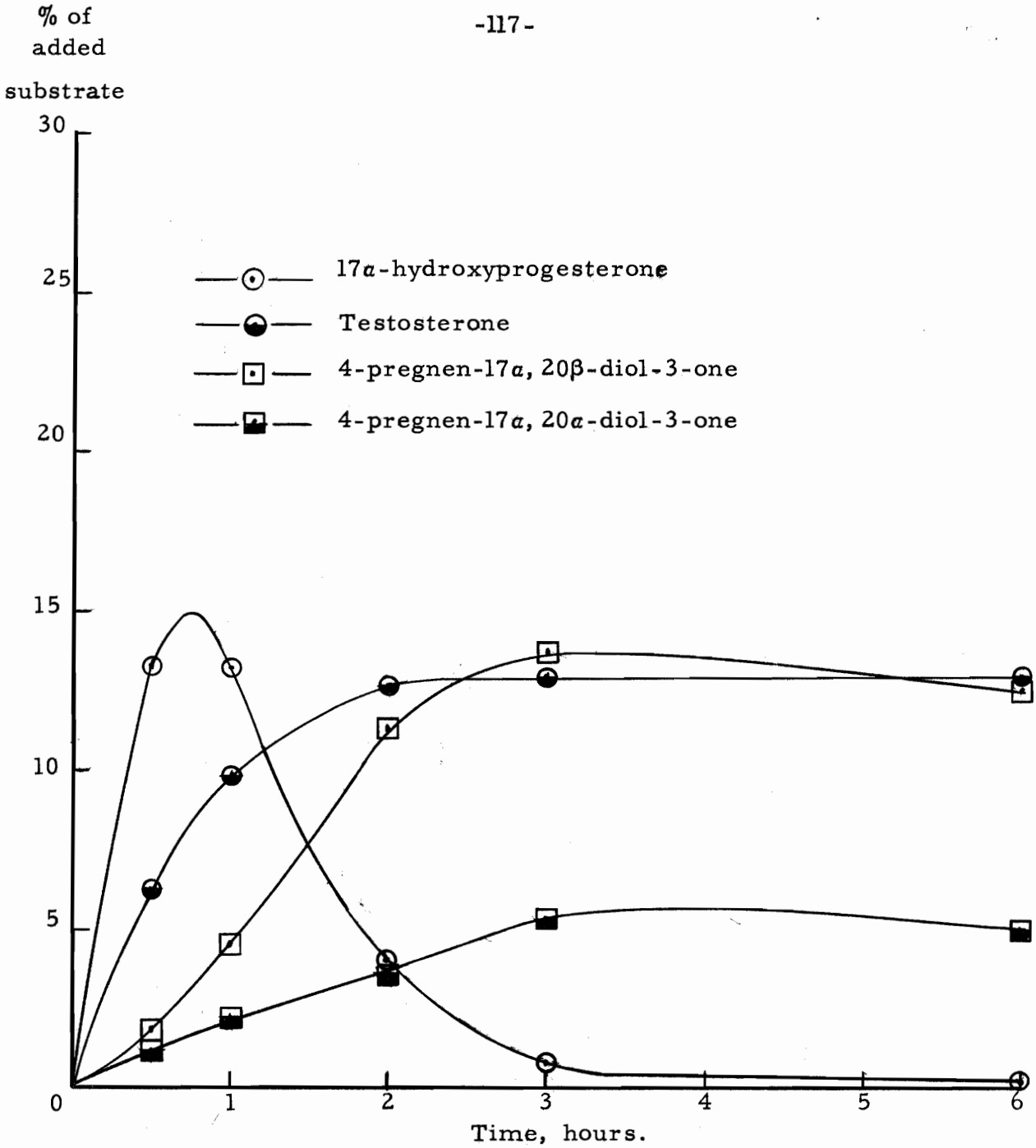


Figure 15. Time course of product formation from progesterone substrate by testis tissue cell-free homogenate.
Substrate: 0.01 μmole; 10 mc/mmole.
Final incubation volume: 10 ml.
Testes from birds stimulated with an 18 hr. photoperiod from Feb. 18-Mar. 16.
Mean testicular weight: 334 mg.

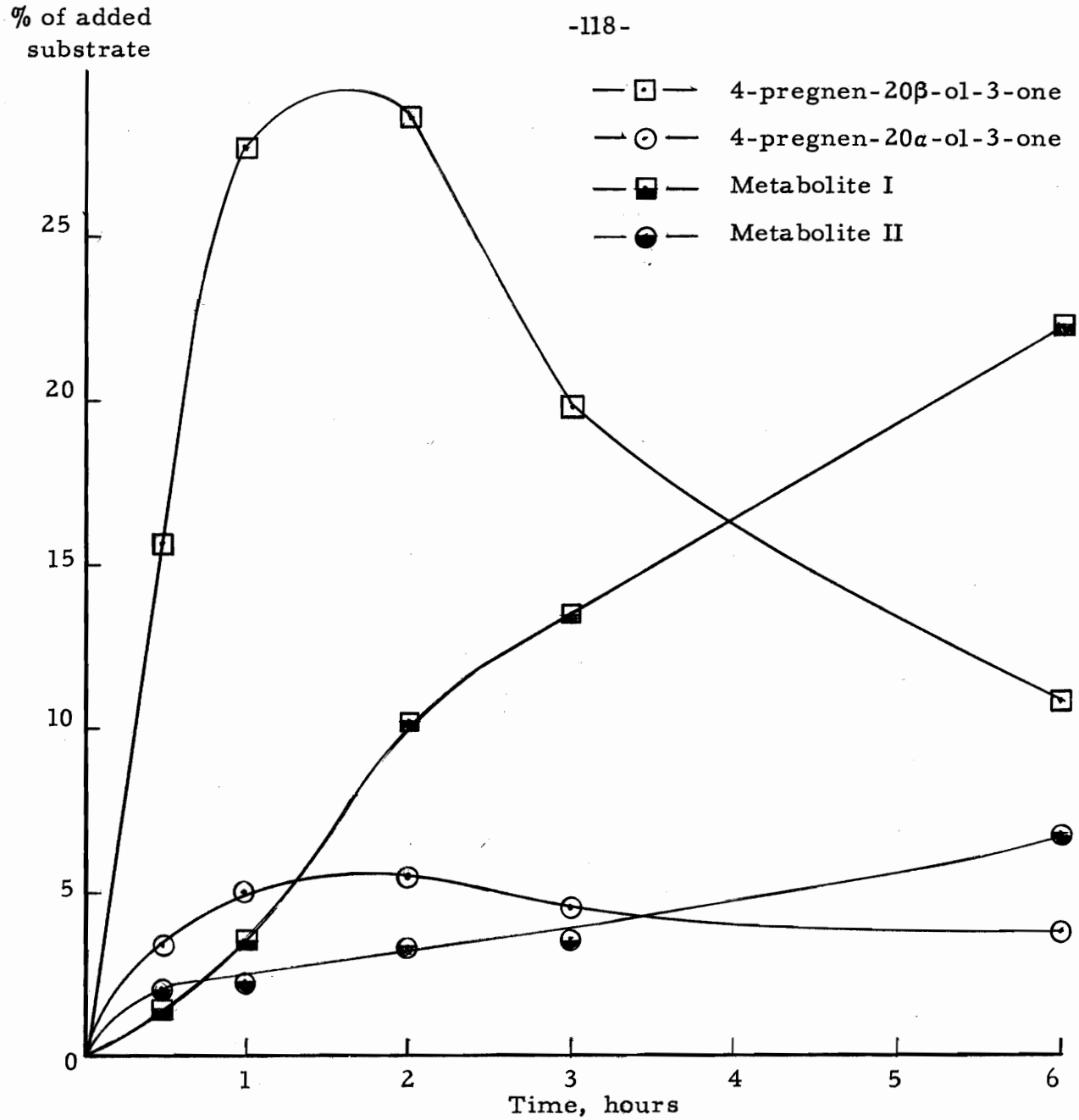


Figure 16. Time course of product formation from progesterone substrate by testis tissue cell-free homogenate.
Testes from birds stimulated with an 18 hr. photoperiod from Feb. 18-Mar. 16.
Mean testicular weight: 334 mg.
Substrate: 0.01 μmole; 10 mc/mmole.
Final incubation volume: 10 ml.

I. Enzyme content. The results from the measurement of the progesterone 20-ketone reductases and 17α-hydroxylase activity in the sparrows testis in various physiological states are presented in Tables XXIV-XXVI.

1. Regressed animals compared with "maximally" light stimulated animals. The first two lines of Table XXIV are data obtained from the birds trapped in December and January and artificially taken through a simulated testicular cycle. At the time of trapping all of the birds had yellow or horn colored beaks. After at least 2 weeks treatment with an 18 hour daily photoperiod, all of these birds had jet-black beaks, indicating endogenous androgen production. They were placed in outdoor aviaries at this time. When the incubation was performed these birds had been outside under the influence of short winter days for 1-2 months. About half had apparently ceased to produce androgens in quantity sufficient to affect the beak coloration, since they possessed partially or entirely yellow beaks. The testicular activity of the rest had apparently not regressed to this stage in that their beaks still showed the effects of endogenous androgens. These birds were therefore divided into two groups, those with yellow beaks and those with black beaks. The testicular weights agreed with the surmise that the birds with yellow beaks had less testicular activity than those with black. (Table XXIV).

A comparison of the enzyme activities of these birds with those artificially stimulated for 5-7 weeks with an 18 hour daily photoperiod shows

Table XXIV. Enzyme activities of the 17α-hydroxylase and progesterone 20-ketone reductases in cell-free homogenates of testicular tissue of birds in various physiological conditions.

Condition	Mean Testicular Weight, mg	Beak Color	Enzyme Activities: mμmoles/mg Protein/Hour			Enzyme Activities: mμmoles/Bird/Hour		
			17α-OHase	20β-red.ase	20α-red.ase	17α-OHase	20β-red.ase	20α-red.ase
Regressed after 2 wk. 18L-6D*	7.2 ± 0.5**	yellow	12.5	2.45	--	8.06	1.58	--
"	14.5 ± 1.9**	black	9.82	3.00	1.02	12.1	3.70	1.25
Trapped Dec.-Jan. 5~~0~~7 weeks 18L-6D	253.	black	1.47	1.78	1.36	21.1	25.5	19.4

* 24 hr photoperiod consisting of 18 hours of light and 6 hours of dark.
** one standard deviation

Table XXV. Enzyme activities of the 17α-hydroxylase and progesterone 20-ketone reductases in cell-free homogenates of testicular tissue of birds in various physiological conditions.

Condition	Mean Testicular Weight, mg	Beak Color	Enzyme Activities: mμmoles/mg Protein/Hour 17α-OHase	20β-red.ase	20α-red.ase	mμmoles/Bird/Hour 17α-OHase	20β-red.ase	20α-red.ase
Regressed after 2 wk. 18L-6D*	7.2 ± 0.5***	yellow	12.5	2.45	--	8.06	1.58	--
Normal, trapped on Feb. 6-8	13.1 ± 3.2	black	9.19	3.57	1.54	9.39	3.65	1.57
Normal, trapped on Mar. 9-11	99.2	black	3.56	1.29	0.38	19.4	7.04	2.05
Trapped Dec.-Jan. 5-7 weeks	283.	black	1.93	2.52	1.85	29.4	38.5	28.3
18L-6D stimulated	384.	black	1.57	1.31	1.03	31.3	26.2	20.6
Normal, trapped on April 17	480.	black	1.08	3.04	1.63	24.3	68.4	36.7

* 24 hr photoperiod consisting of 18 hours of light and 6 hours of dark.
** injected with 500 IU per day from 12-14 through 12-24 and with 250 IU per day from 12-25 through 12-28.
*** one standard deviation.

Table XXVI. Enzyme activities of the 17α-hydroxylase and progesterone 20-ketone reductases in cell-free homogenates of testicular tissue of birds in various physiological conditions.

Condition	Mean Testicular Weight, mg	Beak Color	Enzyme Activities					
			mμmoles/mg Protein/Hour			mμmoles/Bird/Hour		
			17α-OHase	20β-red.ase	20α-red.ase	17α-OHase	20β-red.ase	20α-red.ase
HCG* stimulated	85.6	black	6.61	2.83	0.88	36.0	15.4	4.79
Normal, trapped on Mar. 9-11	99.2	black	3.56	1.29	0.38	19.4	7.04	2.05
Normal, trapped on Feb. 6-8	13.1	black	9.19	3.57	1.54	9.39	3.65	1.57
Trapped Dec.-Jan. 5-7 weeks 18L-6D**	283.	black	1.93	2.52	1.85	29.4	38.5	28.3

* Injected with 500 IU per day from 12-14 through 12-24 and with 250 IU per day from 12-25 through 12-28.

** 24 hour photoperiod consisting of 18 hours of light and 6 hours dark.

that on a protein basis the 17α-hydroxylase activities decreased by a factor of 7-10, while the 20-reductase activity decreased by not more than a factor of 2. However, calculated on the basis of enzyme content per average testicular weight of the birds in each group, the 17α-hydroxylase increased by a factor of 3-4 while the 20-reductases increased by a factor of 15-23.

The net dpm of the 20-reduced isomers measured ranged from 2,000 to 25,000 and 9,000 to 40,000 for the 15 and 30 minute incubations respectively. The net dpm used to calculate 17α-hydroxylase activity ranged from 9,000 to over 100,000 with the various preparations.

2. Normal spring animals compared with artificialy light stimulated animals. Lines 2, 3, and 6 of Table XXV show results from incubation of birds in a natural physiological state at different months of the year. These birds had received no artificial stimulation prior to the experiments. The results seem to fit fairly well into the pattern seen in the regressed and "maximally" light stimulated birds discussed (Table XXIV). Thus, the February birds, whose testicular weight did not differ significantly from the regressed birds with black beaks, had 17α-hydroxylase and 20-reductase levels very similar to these latter birds both on a protein and bird basis. With a 7-8 fold increase in testicular weight over the February birds, the March birds show a 60% decrease in 17α-hydroxylase activity per mg protein accompanied by 54 and 75% decreases in the 20β and 20α-reductases

respectively. The enzyme levels per bird increased by a factor of 2 for the 17α-hydroxylase and 20β-reductase and a factor of 1.3 for the 20α-reductase.

Between March and April a large increase in testicular size was observed (Table XXV). From 99 mg the mean weight increased to 480, or almost a five fold change. The 17α-hydroxylase activity continued to decrease while the reductases increased to approximately the February level on a protein basis. In effect the 17α-hydroxylase activity per bird increased very little - 20% - between March and April, whereas the reductase activities increased 10-15 times.

If the changes in the testes of birds stimulated with artificial photoperiods is assumed to be comparable with that occurring normally, the 20-reductases are observed to increase on a bird basis from the 7.2 mg to the 480 mg testes with the possible exception of the 384 mg testis birds. Calculated on a mg of protein basis, these same activities appear to oscillate between values of 1.5 and 3.00 in the case of the 20β-reductase and between 1 and 2 in the case of the 20α-reductase. The 17α-hydroxylase activity drops from 12.5 mμmoles/mg protein/hr in the 7.2 mg testis bird to a value between 1 and 2 for the 250-480 mg testis birds. On a bird basis the 17α-hydroxylase increases gradually from a value of 8 to between 25 and 31.

3. HCG stimulated animals compared with normal and light stimulated animals. Birds injected with HCG had testicular enzyme levels

approximately double that of birds with testes of a comparable size that had been naturally stimulated. (Table XXVI). The 17α-hydroxylase content per mg of protein was 3-5 times as great in the HCG birds as in the "maximally" light stimulated birds, whereas the 20-reductases were approximately the same or slightly less. These figures transposed to a bird basis show that with 86 mg testes the HCG stimulated birds had as much or more 17α-hydroxylase than the 250-480 mg light stimulated animals. The 20β-reductase was 1/4 to 1/2 and the 20α-reductase 1/8 to 1/5 that of the light stimulated animals. HCG appears to preferentially stimulate 17α-hydroxylation over 20-ketone reduction.

J. Bio-assay of metabolites. The progesterone metabolites from these incubations were bioassayed by the sparrow beak test. Females or immature males were used as assay animals. Metabolites were not all assayed at the same time. In each separate assay testosterone was used as a standard and progesterone as the blank. Results are shown in Table XXVII. Only testosterone and androstenedione were found to be active as androgens by this criterion. Although this assay is not quantitative, testosterone appeared to be 5-10 times as active as androstenedione.

K. Histological Examination.

1. "Maximally" stimulated by artificial 18 hour photoperiods. Slices of testes from sparrows "maximally" stimulated with artificial photoperiods showed full spermatogenic activity. They were in stage 6 as

Table XXVII. Bio-assay of identified metabolites. Compounds were obtained commercially.

Material Assayed	Daily Dose, ug	N	Response	Duration of Assay, days	Cumulative Dose, ug
4-pregnen-17α,20α-diol-3-one	10	5	neg.	21	210
17α-hydroxy-progesterone	30	3	neg.	19	570
Testosterone	2-3	13	pos.*	10***	20-30
4-pregnen-20α-3-one	10	5	neg.	21	210
Androstenedione	15	3	pos.**	12***	170
4-pregnen-20β-ol-3-one	2	6	neg.	18	36
Progesterone	30	3	neg.	19	570

N = number of animals.
* in 12 of 13 animals.
** in 2 of 3 animals.
*** average of first noticeable response.

described by Bartholomew (100). Interstitial cells were extremely difficult to locate and, if present, must have been completely flattened between the enlarged seminiferous tubules.

2. Regressed after stimulation. Both the birds with black beaks and yellow beaks had testicular tissue which appeared quite similar histologically. The tubules were atrophic with no apparent lumina. Spermatogonia were present and possibly some primary spermatocytes. The birds that still had the black beaks in this group had what were probably degenerating sperm towards the center of the tubules. Interstitial tissue was abundant. These testes were essentially in stage 1-2 of Bartholomew (100).

3. Unstimulated. Testes from sparrows trapped on January 24 were between 1.66 and 10.62 mg in combined weight. Spermatogenically these varied from stage 2 to stage 3 (100). The interstitial tissue was abundant and on a unit area basis was more prevalent in the smaller weight testes than in the larger. It might be noted that from a series of ten birds trapped on this date the yellow to gray beaked birds had an average combined testicular weight of 3.01 mg while the black beaked birds had an average of 9.30 mg of testicular tissue. The 5 birds with yellow or gray beaks had combined testicular tissue weights ranging from 1.66 to 4.77 mg, while the black beaked birds ranged from 7.50 to 10.62 mg. From these weight ranges, 5-7 mg was the approximate

testicular weight at which the yellow or gray beaks became black.

Spermatogenic activity and testicular development from birds trapped on February 6 were essentially the same as the birds trapped on January 24. The combined testicular weight of those testes examined histologically was 10.2 mg.

Birds trapped in mid-March were in stage 4 or 5 with regard to spermatogenesis. The interstitium was small per unit area compared to the regressed or late January birds, but was more abundant than in the "maximally" stimulated animals.

4. HCG stimulated. The degree of stimulation obtained with HCG was variable, as judged by testis size. In four birds injected for a two week period from the last part of January to the first of February, the combined testicular weights ranged from 88 to 335 mg. Spermatogenically these testes varied from stage 4 to 6, although mature sperm were never as abundant as in the fully light stimulated animals. Interstitial tissue appeared to be more prevalent in these birds than in artificially light stimulated or normal birds with comparable testicular weights.

V. DISCUSSION

The investigations of the biosynthetic pathways leading to androgen formation from progesterone have answered two of the original questions posed (cf. p. 2). It has been shown that avian testicular tissue is capable of metabolizing progesterone to two androgenic hormones, testosterone and androstenedione, and that the pathway of this biosynthesis is similar to that reported in mammalian tissue (212).

Furthermore, under these in vitro conditions formation of testosterone is favored over androstenedione (Table XXVIII), and in this respect the English sparrow resembles the guinea pig rather than the rat. Whether this is a species difference in testis content of 17β-hydroxydehydrogenase, or in the capacity of this tissue to reduce triphosphopyridinenucleotide necessary for the reduction of androstenedione, is not shown by these studies. However, since the reduction of the steroid 17-keto group by reduced pyridinenucleotides is thermodynamically favored over the reverse reaction at a neutral pH (243), it would appear that the formation of testosterone from androstenedione merely means that adequate amounts of both the 17β-hydroxydehydrogenase and reduced pyridinenucleotide are present. In the absence of specific information, the failure of this reduction to occur could be due either to lack of reduced nucleotide, enzyme, or both. Lindner and Mann (244) have found that the ratio of androstenedione to testosterone in the bull testis decreases

Table XXVIII. Ratios of testosterone to androstenedione under various incubation conditions. Tissue: cell-free homogenates of English sparrow testes.

Substrate	Conc.*	Incubation Period, hr	Mean Testicular Weight, mg	Stimulation**	Ratio***
Progesterone	0.01	3	41	HCG	15.1
"	"	"	177	L	117
"	"	"	235	L	33.4
"	"	"	291	L	30.3
"	"	"	408	L	40.1
"	0.1	"	257	N	5.4
"	0.4	"	201	L	3.9
"	0.2	0.5	86	HCG	>10****
"	"	"	91	L	6.1
"	"	"	283	L	>5****
17α-hydroxy-progesterone	0.01	3	55	HCG	22.5
Androstene-dione	0.05	"	408	L	38.4
Testosterone	0.037	"	235	L	23.1

* μmoles per 10 ml.
** HCG = human chorionic gonadotropin.
L = 24 hr photoperiod consisting of 18 hr light and 6 hr dark.
N = no artificial stimulation.
*** Testosterone:androstenedione
**** Androstenedione below levels of detection.

with age. In immature calves the ratio was greater than 1:1, whereas mature bulls had a ratio of less than 1:10. This leads to speculation as to whether formation of testicular 17β-hydroxydehydrogenase is part of the maturing process in bulls. Although this question was not specifically investigated in sparrow testes, no large change was noted in the ratio of testosterone to androstenedione and the predominant androgen formed was always testosterone (Table XXVIII).

The pathway of androgen biosynthesis from progesterone in testis tissue from P. domesticus (Figure 13) is not only identical with regard to the sequence of chemical reactions but also in the cofactor requirements to that reported for mammals. Thus, TPNH is required for the hydroxylation of progesterone, the cleavage of the side chain of 17α-hydroxyprogesterone, and the reduction of androstenedione to testosterone in mammalian tissue (212), and the only cofactor shown to be necessary for this series of reactions in sparrow testicular tissue was also TPNH. The occurrence of the direct formation of testosterone from progesterone as found by Forchielli et al. (217) has not been completely ruled out in these experiments. It would appear from the results of the 17α-hydroxyprogesterone and androstenedione trap experiments (Table XXII) that less than 6.7 per cent of the testosterone could have been formed directly from progesterone. However, it is possible that the large amount of unlabeled 17α-hydroxyprogesterone trap could have inhibited the

enzyme(s) responsible for this direct formation of testosterone. The second possible alternate pathway of androgen formation would be through 17α-hydroxylation of pregnenolone and either formation of 17α-hydroxyprogesterone or dehydroepiandrosterone from 17α-hydroxypregnenolone followed by formation of androstenedione and testosterone (245). Since no precursor prior to progesterone, such as pregnenolone, was used in these studies it is impossible to evaluate the relative importance of these pathways.

The only apparent difference in progesterone metabolism by the avian testis was the variety and extent of the 20-ketone reduction. Both isomers, 4-pregnen-20β-ol-3-one and 4-pregnen-20α-ol-3-one, have been isolated from human placental tissue (246, 247, 206), human and bovine ovaries (206, 207), and plasma of pregnant women (248). Wiest reported the presence of the 20α-isomer in ovaries and blood of pregnant rats (249), and Short found the same compound in the blood of pregnant sheep and the 20β-isomer in mare placentae (250). Formation of one or both of these reduced compounds has been observed in incubations with ovarian (201, 202, 194), placental (251), fetal testicular (252), and testicular tumor tissue (224, 253). In addition, the presence of 20-ketone reductases has been reported for several non-endocrine tissues (254-260). The first reports of the formation of both 4-pregnen-20β-ol-3-one and 4-pregnen-20α-ol-3-one by normal adult testicular come, however, from the investigations

of Bryson and Sweat using mouse testes (224) and the present studies (261). That this reduction was an activity relatively specific to sparrow testicular tissue was shown by the failure of pancreas or skeletal muscle of the bird to carry out similar reductions under identical conditions. Therefore, it is possible that these two reductases are concerned with testicular function in the bird and are not merely non-specific reductases, as has been suggested by Sweat et al. (258). These workers conducted five day incubations of progesterone with human tissue culture fibroblasts. It is rather difficult to compare this type of work with other tissue incubations, since the amount of tissue used is given in cell counts rather than in weight or protein content. However, if the length of time incubated was necessary to obtain the observed degree of progesterone-20-ketone reduction, the relative activity of the reductases appears much less than that in sparrow testes. Perlman et al. (259) have also reported these activities in mouse fibroblasts of non-glandular origin. These latter investigators used a 3 day incubation period, however, and got only 20% conversion of progesterone to the reduced products. It might also be pointed out that the ratio of bovine pancreas tissue to progesterone substrate used by Nabors and Berliner (256) to demonstrate the reduction to 4-pregnen-20β-ol-3-one was more than 10 times that used in these studies and could explain the negative results obtained with sparrow pancreas tissue. Acevedo et al. (252) reported 20-keto reduction of progesterone to both the α- and β-ols in a

ratio of 7:1 by 24 week old human fetal testes, and Dominguez (253) was able to detect small amounts of 20α reduction of progesterone by human testicular tumor tissue, although formation of the 20β-isomer was not observed. Histologically there was some doubt whether this tumor was adrenocortical or interstitial cell in origin. Balfour et al. (262) have shown that 4-pregnen-20α-ol-3-one is produced and secreted by the calf adrenal until about the 60th day post-partum, at which time the secretion of progesterone replaces that of the reduced compound.

If the assumption is made that the ovaries, testes, and adrenals are capable of producing one or both of the 20-keto reduction products of progesterone, the obvious question is "Why?" Zander and Forbes postulated that these products were physiologically significant gestagens in the human female, on the basis of their biological activity in the Clauberg and Hooker-Forbes tests (248). However, Forbes pointed out that activity in the Hooker-Forbes test did not necessarily have anything to do with the progestational potency of a compound, and recently Wilcox and Wiest (263) have reported that 4-pregnen-20α-ol-3-one is incapable of supporting the decidual reaction in ovariectomized mice, which is, perhaps, a more critical test of progestational activity.

Even if the 20-ketone reduction products are active progestational hormones, this does not explain the reason for the secretion of the 20α-isomer by the adrenal gland of calves or for the production of both the

20α- and 20β-isomers by English sparrow testicular tissue. Since neither 4-pregnen-20α-ol-3-one or 4-pregnen-20β-ol-3-one is androgenic in the sparrow beak assay, the only obvious explanation for the existence of enzymes producing such products is to inactivate excess amounts of testicular progesterone to prevent the secretion of a progestational compound in a male animal. The results presented in Figure 14 lend support to this hypothesis in that the 17α-hydroxylase system is saturated prior to saturation of the 20-ketone reductases. This regulatory explanation could hold true for the calf adrenal and ovarian reduction of the 20-ketone also, although the reason for a change from 4-pregnen-20α-ol-3-one to progesterone secretion by the calf adrenal at ca. 60 days of age is a little difficult to see. Presumably this change could indicate a transition period in which progesterone metabolism is shifting from inactivation to corticoid synthesis. This is pure speculation, however, and involves too many postulates to be very likely. Thus, there would have to be a decrease in the 20-ketone reductase followed, after a lag period, by increases in the 17α- and 20-hydroxylases to explain the progesterone secretion in the interim. If this were true, it would be expected that, after this lag period, secretion of progesterone by the adrenal would decrease due to increased utilization for corticoid biosynthesis. Balfour et al. (264) have found that adult bulls and non-pregnant cows have adrenal venous levels of progesterone that are 1/4 to 1/5 that of 60-70 day old calves. Since the 17α-hydroxylase

levels are highest relative to the 20-ketone reductases in the smallest sparrow testes examined, and this ratio decreases as testis size and sexual activity increase, it would not be possible to postulate a similar function of the 20-ketone reductase in this species as in the calf.

Because the evidence for progesterone production by avian testes is virtually non-existant, it would be interesting to know if the avian adrenal also secretes progesterone, and if this progesterone could serve as precursor of testicular androgens. By a consideration of dilution factors, the small percentage of the arterial circulation which goes to the spermatic arteries, and the reduction of progesterone by other tissues, especially the liver, the possibility of such an interrelationship between endocrine glands seems improbable.

The formation of 4-pregnen-17α, 20α-diol-3-one and 4-pregnen-17α, 20β-diol-3-one is somewhat more uncommon than the formation of the 20-reduced progesterones. This reduction takes place in liver after ring A reduction (254), and the 20β-isomer has been reported to be formed by mammalian testicular tissue after incubation with progesterone (212). Sandor and Lanthier (203) found both isomers after incubation of human ovarian slices with 17α-hydroxyprogesterone. Recently Dominquez has reported the formation of both isomers by human testicular tumors in vitro with either progesterone or 17α-hydroxyprogesterone as substrate (253). But in the case of the 20α-isomer the rate of formation was

greater from progesterone than from 17α-hydroxyprogesterone, indicating that the 20-ketone reduction probably occurred prior to 17α-hydroxylation, whereas the formation of 4-pregnen-17α, 20β-diol-3-one was greater from 17α-hydroxyprogesterone than from progesterone. In the sparrow testes comparative rates (Table XXI) indicated that 17α-hydroxylation preceded 20-ketone reduction in the formation of both of these diols from progesterone. Acevedo et al. (252) have also found these two compounds produced from progesterone by fetal testicular tissue incubations; two and one half times as much of the 20α- isomer was formed as the 20β.

There are only two instances to this author's knowledge of the isolation of 4-pregnen-17α, 20α-diol-3-one or 4-pregnen-17α, 20β-diol-3-one from blood. Idler (265) reported the isolation of the 20β-isomer from peripheral blood of salmon (Oncorhynchus nerka) and Dominguez (253) published tentative evidence for the occurence of the 20α-isome r in spermatic vein blood of a patient with bilateral testicular tumor. In the case of the salmon, reduction of the 20-ketone by peripheral tissues after secretion of 17α-hydroxyprogesterone is a distinct possibility.

The possible function of these compounds is not known. The 20α-isomer was androgenically inactive in the sparrow beak assay. This does not preclude some unknown hormonal action. It is possible that this 20-ketone reduction is a second regulatory valve to prevent overproduction of androgens. However, since the 20α-isomer was not appreciably metabolized to any

other product by sparrow testicular tissue (cf. p.112), the reduction of 17α-hydroxyprogesterone to this isomer appears to be a physiologically irreversible reaction. If this is the case, it would seem that the sparrow testis would have to secrete 4-pregnen-17α, 20α-diol-3-one and possibly the 20β-isomer. This would be necessary to prevent inhibition of the side chain splitting enzyme as occurred in one of the trap experiments (Table XXII). On the other hand, Marshall (266) reported that the interstitial and tubule cells of bird testes appear completely "exhausted" at the end of the breeding cycle and feels that, to some extent, there is an inherent testicular cycle of activity dictated by this "exhaustion" of testicular elements. He also postulates that the refractory phase of the cycle is not just necessary for pituitary recovery, but also for regeneration of these testicular elements. It is interesting to speculate that if 4-pregnen-17α, 20α-diol-3-one and its 20β isomer were formed but not secreted by the testes, it is conceivable that these two compounds would build up in the tissue during the course of the breeding season and prevent the production of androgenic hormones completely by inhibition of the formation of androstenedione from 17α-hydroxyprogesterone. This in turn could inhibit the latter stages of spermatogenesis, since there is some evidence for the role of androgenic hormones in this period on sperm formation (267). The final results of such an effect would be regression in testicular size and function. The removal of

progesterone by 20-ketone reduction could have an action here of delaying the build up of 4-pregnen-17α, 20α-diol-3-one and 4-pregnen-17α, 20β-diol-3-one and thereby extending the period of active androgen formation and sperm production. This build up of the 20-ketone reduction products of 17α-hydroxyprogesterone and its effects would probably not be noticed using testicular homogenates.

The question of whether the enzymes reducing the 20-ketone function of progesterone and 17α-hydroxyprogesterone are the same enzymes acting on different substrates or discrete enzymes for each substrate, cannot be unequivocally answered. However, the results of the trap experiments (Table XXIII) seem to indicate that it is likely there are at least three separate reductases present in avian testicular tissue to reduce progesterone and 17α-hydroxyprogesterone to the respective 20α- and 20β-hydroxyl compounds. The presence of one μmole of unlabeled androstenedione in the incubation medium caused a 64% inhibition of the formation of 4-pregnen-17α, 20β-diol-3-one, but a 50% stimulation of 4-pregnen-20β-ol-3-one formation. This is all the more striking when it is realized that there was actually a concomitant 19-20 fold increase in labeled 17α-hydroxyprogesterone accumulation (Table XXII). There is no similar evidence of a difference in the two 20-keto reductases forming the 20α-ol compounds. This does not necessarily mean that these two activities are properties of the same

enzyme, however.

The two most polar metabolites, which were not fully identified are, for this reason, unknown factors in the endocrinology of sparrow testes. From the available data (Table XI) it would appear likely that these two compounds are 20-hydroxy steroids with a second hydroxyl in such a position in the ring that it can be acetylated by the conditions used. It should be pointed out that it has not been established that the Δ^4-3-keto structure is intact in either of these unknowns.

In conclusion, the contributions of this portion of the study are two-fold. First, the biosynthetic scheme of androgens from progesterone proposed for mammalian testis tissues (209, 212) has been extended to a second class of vertebrates. Secondly, reduction of progesterone to 4-pregnen-20α-ol-3-one and 4-pregnen-20β-ol-3-one and 17α-hydroxy-progesterone to 4-pregnen-17α, 20α-diol-3-one and 4-pregnen-17α, 20β-diol-3-one has been shown to occur. This is the first time that formation of all four of these reduction products has been demonstrated in normal adult testis tissue, and the possible role of these reductases in regulating testicular hormone production and thereby, sperm production has been suggested.

The final question asked at the beginning of these studies concerned the variation of the capacity of the avian testes to synthesize androgenic hormones during the annual testicular cycle. The results (Tables XXIV-

XXVI) demonstrate several points in this connection. Although the 17α-hydroxylating capacity of the testes increases on a bird, or average combined testicular weight, basis during the increase in testicular size, an approximate 60 fold increase in testis size was associated with only a 3-4 fold increment in 17α-hydroxylase. On the other hand, the 20-reductases increase 20-40 fold - more closely paralleling the testicular growth. This trend is evident when the results are expressed on a milligram of protein basis also. Whereas the 17α-hydroxylase values decrease from a value of 12.5 to 1.08 as the testis size increases from 7.2 to 480 mg, the 20-ketone reductase activities merely oscillate between values of 1 and 3 or 0.4 to 1.8.

These data raise the question as to cellular locations of the enzymes involved. Certainly, the best available evidence (39-44) indicates that androgenic hormones are synthesized in the interstitial cells of the testes. If it is assumed that the interstitial cells are the location of the 17α-hydroxylase activity, the question is, are the 20-ketone reductases located in these or other cells? If these latter enzymes were located in the interstitial cells, this would mean that gonadotropic stimulation caused more formation or activation of the reductases than of the 17α-hydroxylases in these cells. If the increase in 17α-hydroxylase activity represents an increase in the number of Leydig cells, as Blanchard's (128) and Threadgold's (79) papers appear to support, then the conclusion

would seem inescapable that the hydroxylase and reductases are in separate cell types. If it is then postulated that the progesterone-20-ketone reductases are located in a cellular element of the seminiferous tubules, the changes of these activities with testicular size changes are more logical. Thus, most of the testis size increase can be attributed to augmentation of tubular elements, and a very small percentage to an increase in interstitial cells. The enzyme activities, as mentioned, show the same relative increases, i.e., the 20-ketone reductases increase much more than the 17α-hydroxylase.

Some rational for this theory can be found in the literature. Many histological studies (128, 79, 268) of regressed bird testes have shown that the major component at this time is interstitial tissue. The enzyme picture observed in the present study confirms this, since the 17α-hydroxylase greatly predominated over the 20-reductases in the regressed testes. These regressed gonads might be very similar to x-ray treated testicles in which the total size is reduced 75% but the interstitial tissue and secondary sex characters remain unchanged (42).

It also appears that the content of the two different types of enzyme activities studied shows maximal rates of increase on a bird basis at different stages of testicular development. There is a 100% increase in the 17α-hydroxylase content between February and March, and, as can be seen in Figure 17, the maximal rate of increase probably

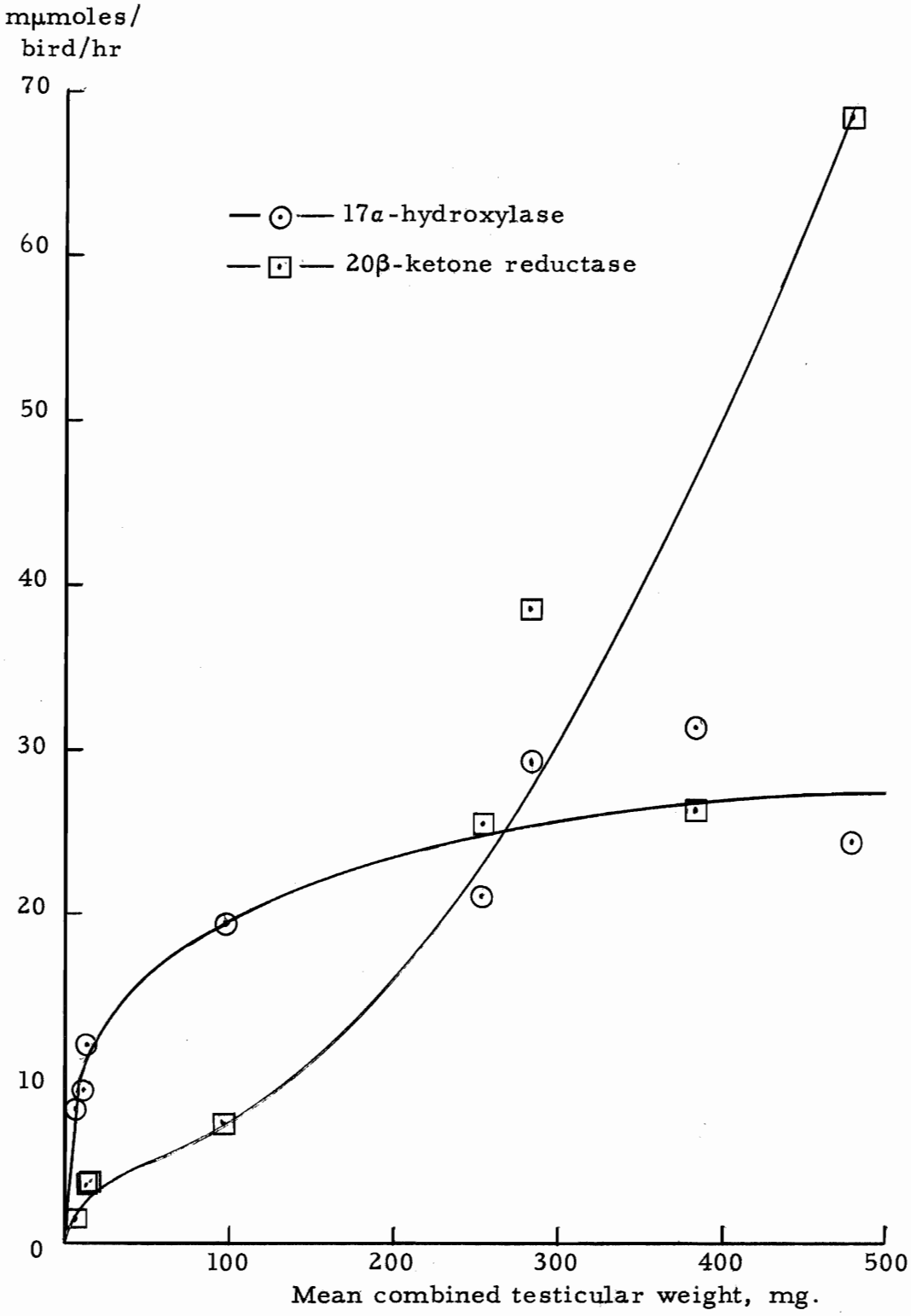


Figure 17. Variation of the 17α-hydroxylase and progesterone 20β-ketone reductase activities in testicular tissue cell-free homogenates with testicular weight.

actually preceded this. The maximal rate of change of the formation of the 20β-ol reduction product appears to be somewhat later, or after the gonads are larger. This can be seen graphically in Figure 17 and by comparing the 99.2 and 253 mg testes 20β-reductase activities (Tables XXIV and XXV).

The differences in 17α-hydroxylase activities are not marked between regressed birds with yellow beaks and the regressed birds with black beaks or the normal birds with black beaks and 13.1 mg testes. This may lead to the conclusion that there is a very strict threshold level of androgen production required for pigment deposition in the beak and that the regressed yellow beak birds we re producing subthreshold levels of androgens, while the others were producing slightly more (Tables XXIV and XXV). That this conclusion is not necessarily true can be seen by the following considerations. First, these so-called yellow beaked birds were not composed solely of animals with completely yellow bills. The beaks of this group ranged from completely yellow to yellow at the base only. Therefore, there was probably significant biological variation within the group. However, it might be noted that the 7.2 mg testicular weight was significantly different from both the 13.1 and 14.5 mg values at a 99% confidence level. Second, there is no assurance that the 17α-hydroxylase is the rate limiting step in androgen synthesis. Although the rate of 17α-hydroxylation appears to

be slower than the rate of side chain splitting in normal testes from July birds (Table XXI), this does not necessarily mean that it is so at all times of the year, nor does it mean that the real physiological rate limiting step could not be prior to progesterone synthesis. However, the observations on changes of secondary sex characters and behavior, and the observations on total 17α-hydroxylation in testes stimulated to varying degrees seem to indicate a general parallelism. Another possible explanation for the pigment deposition of the sparrow beak responding to such a small increment in the 17α-hydroxylase activity is that the sensitivity of the beak to androgens may also be modified by photoperiod as has been shown for the chick comb (269).

The results of HCG stimulation were somewhat surprising. It was expected that this gonadotropin would stimulate primarily the interstitial cells and, therefore, primarily the 17α-hydroxylase activity. However, when the enzyme activities of these birds are compared with the enzyme activities of naturally stimulated birds with similar size testes (Table XXVI), it is seen that all the enzyme activities tested increased on a protein basis. However, compared to birds with testicular weights from 7.2 to 14.5 mg (Table XXIV), the enzyme activities of HCG treated birds still decreased per mg of protein, but not as much as the light stimulated animals. On a bird basis the 17α-hydroxylase activity of HCG stimulated testes was greater than that of testes five

times as large, whereas the 20-ketone reductase activities were only 1/3-1/5 as great as those of the larger, light stimulated gonads. The greater reductase content of the HCG testes over similar size testes from birds trapped in March could correlate histologically with a greater level of spermatogenic activity in the HCG stimulated birds. In these birds all tubule lumina were open and mature sperm could be found in some. In the 99.1 mg testes from birds trapped in March, most of the tubules contained no lumen, and no mature sperm were observed. The explanation for this fairly great spermatogenic stimulating activity of a supposedly primarily LH simulating hormone (52) is not known. It is possible, however, that using such a high dose of a relatively crude preparation the effects of other, contaminating urinary gonadotropins were observed.

The inherent difficulties and limitations of simulataneous measurement of two or three enzyme activities that compete for the same substrate should be pointed out. With regard to the 17α-hydroxylase, Table II shows that the activity appeared fairly proportional over a two fold range of enzyme concentration at the substrate level used. However, in Table XXV it is seen that the birds with regressed testes had 8-10 times as much 17α-hydroxylase per mg of protein as the stimulated birds. Since the product formation was measured at 3 time intervals, 0, 15, and 30 minutes, and the rate of 17α-hydroxylation invariably decreased

during the 15-30 minute interval (Figure 10), the straight line used to calculate the activities was drawn between the 0 and 15 minute points. There is, therefore, no guarantee that the rate of 17α-hydroxylation, especially at the high enzyme levels, did not start to decrease prior to the arbitrary 15 minute determination. However, since the rate of 20-ketone reduction, as measured from the rate of product formation during the 15-30 minute interval, either increased or remained constant at the low testicular weights (Table XXV) when the 17α-hydroxylase had increased by a factor of 8-10, it is unlikely that there was actually a limiting amount of substrate for the 17α-hydroxylase. This is even more evident when it is recalled that the "apparent Michaelis constant" of this enzyme is lower than that of either 20-ketone reductase (Figure 14). The fact that the rate of 17α-hydroxylation decreased during the 15-30 minute interval while the rates of 20-ketone reduction are actually increasing seems somewhat anomolous unless the substrate is bound by the reductases prior to the 15-30 minute interval (Figures 8 and 9) and is thus made unavailable to the 17α-hydroxylase. A second possible explanation would be partial inactivation of the hydroxylating enzyme during the second 15 minute interval. In conclusion it may be said that there is some danger in measuring activities of two or more enzymes competing for the same substrate, and therefore the highest values of activity per mg of protein in Tables XXIV-XXVI must be

looked upon as minimal values. However, since the 20-ketone reductase activities did not decrease on a mg of protein basis from the stimulated to non-stimulated testes, and these activities were measured at the 15-30 minute interval, it is improbable that substrate was limiting in any of these measurements.

The observed decrease in total 20-ketone reduction of progesterone and increase in total 17α-hydroxylation with a decrease in the incubation temperature from 41^{o} C to 37^{o} C (Table X) is interesting to contemplate in light of Riley's observations on spermatogenic activity in P. domesticus testes (270). It was noted that active spermatogenesis occurred only in the early morning hours, a time when the body temperature was lowest, about 40^{o} C. If it is assumed that androgen formation is required for the latter stages of spermatogenesis (267), then it is conceivable that there is a relationship between increased androgen production and increased spermatogenic activity. It is obvious that this situation differs from that in mammals in which 37.5^{o} C is a sufficiently high temperature to completely inhibit spermatogenesis.

The results of the measurement of enzyme content of the sparrows' testis at different periods of testicular activity indicate that the 17α-hydroxylase, an enzyme concerned with the biosynthesis of androgenic hormones, increases very little compared to the increase in testicular size. The enzyme activities that catalyze the reduction of the 20-ketone

of progesterone increase at a rate more nearly that of the testis. It has been suggested that the 17α-hydroxylase is located in the interstitial cells, while the 20-ketone reductases are in cells of the seminiferous tubules.

VI. SUMMARY

It has been shown by in vitro incubations that cell-free homogenates of testicular tissue from the English sparrow (Passer domesticus) are capable of synthesizing androgenic steroid hormones from 4-pregnen-3, 20-dione-4-C^{14} by a pathway involving 17α-hydroxylation, cleavage of carbons 20 and 21 to form 4-androsten-3,17-dione, and reduction of the 17-ketone to form 4-androsten-17β-ol-3-one. The only exogenous cofactor required for this sequence of reactions is reduced triphosphopyridinenucleotide. In addition, both 4-pregnen-3, 20-dione, and 4-pregnen-17α-ol-3, 20-dione are reduced at the 20-ketone to form 4-pregnen-20α-ol-3-one, 4-pregnen-20β-ol-3-one, 4-pregnen-17α, 20α-diol-3-one, and 4-pregnen-17α, 20β-diol-3-one. Two other products of progesterone metabolism believed to be 4-pregnen-20β-ol-3-one and 4-pregnen-20α-ol-3-one with a second hydroxyl at a position in the steroid nucleus have been incompletely identified.

The variation of the testicular content of the 17α-hydroxylase and the two progesterone-20-ketone reductases over a wide range of natural and artificial testicular stimulation was determined. It was found that on a milligram of protein basis the 17α-hydroxylase decreased by a factor of 10 with an increase in testis weight from 7.2 to 480 milligrams, whereas the 20-ketone reductases remained relatively constant or decreased to a much lesser extent, by a factor of two, over similar testicular weight changes. Calculated on the basis of enzyme content per bird by use of

an average combined testicular weight, the total testicular 17α-hydroxylase increased by a factor of three to four, while the 20-ketone reductases increased by a factor of twenty to forty when the mean testicular weight increased from 7.2 to ca. 500 miligrams.

Artificial testicular stimulation with human chorionic gonadotropin stimulated both the 17α-hydroxylase and 20-ketone reductases, but appeared to have a greater effect on the 17α-hydroxylase.

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